Solange Cristina Bastos da Costa

OCCUPATIONAL EXPOSURE TO FORMALDEHYDE.

GENOTOXIC DAMAGE AND SUSCEPTIBILITY EVALUATION IN ANATOMICAL PATHOLOGY LABORATORY WORKERS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Doutor João Paulo Teixeira

Categoria – Investigador Auxiliar

Afiliação – Instituto Nacional de Saúde Doutor Ricardo Jorge

Co-orientadora – Doutora Beatriz Porto

Categoria – Professora Auxiliar

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

The experimental work presented in this thesis was performed at the:

- Environmental Health Department of the National Institute of Health, Porto, Portugal;
- Department of Nutrition of the Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway;
- Toxicology Unit of the Department of Psychobiology, University of A Coruña, A Coruña, Spain;
- Toxicology Laboratory and Farmacognosia Laboratory of the Faculty of Pharmacy, University of Porto, Porto, Portugal;
- Department of Genetics of the Faculty of Medical Sciences, New University of Lisbon, Lisbon, Portugal.

Statistical analysis was performed at the Toxicology Unit of the Department of Psychobiology, University of A Coruña, Spain.

This work was supported by the Portuguese Foundation for Science and Technology (FCT) under the grant SFRH/BD/46929/2008, under the QREN - POPH - Type 4.1 - Advanced Training, subsidized by the European Social Fund and national funds from the MCTES.

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

.

QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL
PORTUGAL 2007.2013

POPH
PROGRAMA OPERACIONAL POTENCIAL HUMANO

UNIÃO EUROPEIA
Fundo Social Europeu

CONTENTS

ORIGINAL PUBLICATIONS

The present work contains techniques and/or data also presented in the following scientific papers:

- **Costa S**., García-Lestón J., Coelho M., Coelho P., Costa C., Silva S., Porto B., Laffon B., Teixeira J.P. (2013) Cytogenetic and immunological effects of formaldehyde in a group of exposed workers. Journal of Toxicology and Environmental Health Part A 76:217–229.

- **Costa S**., Brandão F., Coelho M., Costa C., Coelho P., Silva S., Porto B., and Teixeira J.P. 2013. Micronucleus frequencies in lymphocytes and buccal cells in formaldehyde exposed workers. WIT Transactions on Biomedicine and Health, 16: 83-94.

- **Costa, S**., Pina, C., Coelho, P., Costa, C., Silva, S., Porto, B., Laffon, B. and Teixeira, J.P. 2011. Occupational exposure to formaldehyde: genotoxic risk evaluation by comet assay and micronucleus test using human peripheral lymphocytes. Journal of Toxicology and Environmental Health Part A, 74(15-16): 1040-1051.

- **Costa, S**., Coelho, P., Costa, C., Silva, S., Mayan, O., Santos, L.S, Gaspar, J.and Teixeira, J.P. 2008. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. Toxicology, 252(1-3): 40-48.

- Ersson, C., Møller P.; Forchhammer, L.; Loft, S., Azqueta, A., Godschalk, R.W.L., van Schooten, F.J., Jones, G.D., Higgins, J.A., Cooke, M.S., Mistry, V., Karbaschi, M., Phillips, D.H., Sozeri O, Routledge, M.N., Nelson-Smith, K., Riso, P., Porrini, M., Matullo, G., Allione, A., Stepnik, M., Ferlińska, M., Teixeira, J.P., **Costa, S**., Corcuera, L.A. López de Cerain, A., Laffon, B., Valdiglesias., V., Collins, A., Möller, L. 2013. An ECVAG inter-laboratory validation study of the comet assay: inter-laboratory and intra-laboratory variation of DNA strand breaks and FPG-sensitive sites in human mononuclear cells. Mutagenesis, 28: 279–286.

- Forchhammer, L., Ersson, C., Loft, S., Möller, L., Godschalk, R.W.L., Jones, G.D., Higgins, J.A., Cooke, M., Mistry, V., Karbaschi, M., Collins, A.R., Azqueta, A., Phillips, D.H., Sozeri, O., Routledge, M.N., Nelson-Smith, K., Riso, P., Porrini, M., Matullo, G., Allione, A., Stępnik, M., Komorowska, M., Teixeira, J.P., **Costa, S**., Corcuera, L.A., López de Cerain, A., Laffon, B., Valdiglesias. V., Møller P. 2012. Inter-laboratory variation in DNA damage using a standard comet assay protocol. Mutagenesis, 27: 665-672.

- Lorenzo, Y., **Costa, S**., Collins, A.R., Azqueta, A. 2012. The comet assay, DNA damage, DNA repair and cytotoxicity: Hedgehogs are not dead. Mutagenesis 28(4):427-32.

- Forchhammer, L., Johansson, C., Loft, S., Möller, L., Godschalk, R., Langie, S., Jones, G.D., Kwok, R.W., Collins, A.R., Azqueta, A., Phillips, D.H., Sozeri, O., Stepnik, M., Palus, J., Vogel, U., Wallin, H., Routledge, M.N., Handforth, C., Allione, A., Matullo, G., Teixeira, J.P., **Costa, S**., Riso, P., Porrini, M., Møller, P. 2010. Variation in the measurement of DNA damage by comet assay measured by the ECVAG inter-laboratory validation trial. Mutagenesis, 25: 113-123.

And book chapters:

- **Costa, S**., and Teixeira, J. P. 2013. Toxicology. In *Encyclopedia of Toxicology*, 3rd Edition, Elsevier (*in press*). DOI: 10.1016/B978-0-12-386454-3.00440-1
- **Costa, S**., and Teixeira, J. P. 2013. Comet Assay. In *Encyclopedia of Toxicology*, 3rd Edition, Elsevier (*in press*). DOI:10.1016/B978-0-12-386454-3.00672-2

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase	**MN**	micronuclei
ALDH	aldehyde dehydrogenases	**MNB**	micronuclei in buccal cells
BMCyt	buccal micronucleus cytome assay	**MNL**	micronuclei in lymphocytes
BM	buccal mucosa	**NAD^+**	nicotinamide adenine dinucleotide
BNbud	nuclear buds in buccal cells	**NADPH**	nicotinamide adenine dinucleotide phosphate
CAs	chromosomal aberrations	**Nbud**	nuclear buds
CBMN	cytokinesis-block micronucleus assay	**OECD**	Organisation for Economic Co-operation and Development
CD	clusters of differentiation	**%TDNA**	percentage DNA in tail
$CD3^+$	total T cells	**PBLs**	peripheral blood lymphocytes
$CD4^+$	T-helper cells	**ROS**	reactive oxygen species
$CD8^+$	T-cytotoxic cells	**SCE**	sister chromatid exchange
$CD19^+$	B cells		
$CD16^+56^+$	natural killer cells	**SCGE**	single cell gel electrophoresis
CSAs	chromosome-type aberrations	**SD**	standard deviation
CTAs	chromatid-type aberrations	**SE**	Standard error mean
DSB	double strand break	**SSB**	single strand break
EH	environmental health	**SNPs**	single nucleotide polymorphisms
EU	European Union	**Tc**	T-cytotoxic cells
FA	formaldehyde	**TCR**	T Cell Receptor
FDH	glutathione-dependent formaldehyde-dehydrogenase	**TCR-Mf**	TCR mutation frequency
FEMA	US Federal Emergency Management Agency	**Th**	T-helper cells
FEV	forced expiratory volume	**TWA**	time-weighted average
FISH	fluorescence in situ hybridization	**US**	United States of America
FVC	forced vital capacity	**WHO**	World Health Organization
GSH	glutathione		
HR	homologous recombination		
IARC	International Agency for Research on Cancer		
MHC	major histocompatibility complex		

ABSTRACT

Formaldehyde (FA) is a high-volume production chemical produced worldwide with a large range of industrial and medical uses. At room temperature is a flammable and colourless gas with a strong pungent odour. Given its economic importance and widespread use, many people are exposed to FA both environmentally and/or occupationally. Over the last two decades, several epidemiological studies have revealed an increased risk of cancer development among workers exposed to FA, namely nasopharyngeal cancer and myeloid leukaemia. Based on these findings plus supporting evidence from animal studies and data on mechanisms of carcinogenesis FA status was recently revised and classified as a human carcinogen. FA's genotoxicity is confirmed in a variety of experimental systems ranging from bacteria to rodents. However, data from human studies is conflicting and needs further investigation in particular the biological evidence of FA's ability to induce (geno)toxicity on distant-site tissues. In addition, published studies on the immunological effects of FA indicate that this compound may be able to modulate immune responses, although data in exposed subjects are still preliminary. The highest level of human exposure to FA occurs in occupational settings. Several studies point to pathology and anatomy laboratories as one of the occupational settings where workers are frequently exposed to levels of FA near or superior to recommended limit values.

The aim of the present study was to evaluate the occupational exposure to FA in a multistage approach relating the exposure with different biomarkers (dose and effect) and individual susceptibility. Eighty-five workers from hospital anatomical pathology laboratories exposed to FA and eighty-seven non-exposed controls took part in the study. Air monitoring was performed in worker's breathing zone for representative working periods and the level of FA-exposure in workplace air was estimated. Formic acid in urine was investigated as a potential biomarker of internal dose for FA occupational exposure. Genotoxicity was evaluated in peripheral blood lymphocytes (PBLs) by means of cytogenetic alterations (chromosomal aberrations, CAs; micronucleus, MN; sister-chromatid exchange, SCE), DNA damage (comet assay, percentage of tail DNA, %TDNA) and T-cell receptor (TCR) mutation assay. The frequency of MN in exfoliated buccal cells a first contact tissue was also assessed. Percentages of different lymphocyte subpopulations were selected as immunotoxicity biomarkers. In addition, the effect of polymorphic genes of xenobiotic metabolising enzymes (*CYP2E1*, *GSTM1, GSTT1, GSTP1*) and DNA repair enzymes (*FANCA, RAD51, XRCC2, XRCC3, XRCC1, PARP1, MUTYH, BRIP1*) on the endpoints studied were determined.

The mean level of FA-exposure was 0.38±0.03 ppm, exceeding recommended limit values, as observed in other studies. The concentration of formic acid in urine increased significantly in workers compared to controls, but no significant correlation was found with the FA-level of exposure. All cytogenetic endpoints and DNA damage evaluated by comet assay were significantly increased in PBLs of FA-exposed workers compared to controls. Further, exposed workers showed higher frequencies of MN formation in both PBLs and exfoliated buccal cells. A significant positive correlation was found between MN frequency in these two tissues and FA-levels of exposure and duration, which confirms MN sensitivity to evaluate the genotoxic action of FA in occupational exposed subjects. The exposed group also presented significant alterations in the percentage of cytotoxic T lymphocytes, natural killer (NK) cells and B lymphocytes in comparison with control group. Concerning the effect of susceptibility biomarkers on the different endpoints studied, results suggest that polymorphisms in *CYP2E1*, *GSTP1* and *GSTM1* metabolic genes are associated with increased genetic damage in FA-exposed subjects. Furthermore, a polymorphic gene involved in FANC repair pathway, *FANCA*, significantly altered the level of genetic damage induced by FA exposure, revealing a potential novel repair pathway involved in the repair of genetic lesions caused by FA-occupational exposure.

Data obtained in the present study show that workers in anatomical pathology laboratories are exposed to air-levels of FA greater than recommended criteria, indicating a potential risk to workers' health. Further, FA-exposed workers presented higher levels of genetic damage compared to controls. Genotoxic endpoints analysis are of great interest in risk assessment of occupational carcinogens because they precede by a long time the potential health effects, thus offering a greater potential for preventive measures. The results of the present biomonitoring study emphasise the need of more effective measures in order to protect the workers from potentially hazard health effects due to occupational exposure to FA. Implementation of security and hygiene measures, such as periodic air sampling and medical surveillance, as well as good practice campaigns may be crucial to decrease risk. The obtained information may also provide new important data to be used by health care programs and by governmental agencies responsible for setting the acceptable levels for occupational exposure to FA.

RESUMO

O Formaldeído (FA) é um dos compostos químicos mais utilizados em todo o mundo, apresentando-se, em condições normais de pressão e temperatura, como um gás incolor de odor característico e intenso. A sua aplicação é multifacetada e transversal a praticamente todas as atividades, sendo que a exposição humana a este composto assume particular importância a nível industrial e no sector da saúde, onde é bastante utilizado. Ao longo das últimas duas décadas vários estudos epidemiológicos revelaram uma associação entre a exposição ocupacional a este aldeído e incidência de cancro nasofaríngeo e leucemia (do tipo mieloide) entre trabalhadores do sector industrial e da saúde. Com base nestes estudos e em dados obtidos em ensaios experimentais com animais o FA foi recentemente reclassificado agente carcinogénico humano pelas principais agências mundiais. O potencial genotóxico do FA está largamente descrito tanto *in vitro* como *in vivo* desde bactérias até roedores. No entanto, em estudos de biomonitorização humana, o caracter genotóxico do FA é inconclusivo, e carece de mais investigação em especial no que concerne ao seu efeito em tecidos distantes do local de absorção. Por outro lado, alguns autores reportaram que o FA poderá influenciar parâmetros imunológicos e por conseguinte afetar a resposta imunitária. Vários estudos apontam os laboratórios de anatomia patológica como sendo um dos cenários ocupacionais em que os trabalhadores estão expostos a elevados níveis de FA. Nestes laboratórios decorrem atividades de rotina que implicam a libertação de vapores de FA, com consequente inalação por parte dos trabalhadores, pela utilização de formol usado em procedimentos anatomopatológicos para conservar e armazenar biópsias e peças cirúrgicas.

O objetivo do presente estudo foi avaliar a exposição ocupacional a FA em profissionais dos Serviços Hospitalares de Anatomia Patológica, utilizando para isso uma abordagem múltipla de modo a relacionar diferentes tipos de biomarcadores de dose, efeito e suscetibilidade. A integração dos resultados dos diferentes biomarcadores estudados permitir-nos-á investigar a relação entre a exposição ao FA e possíveis efeitos biológicos adversos, consequentes da exposição profissional a este composto.

Para o efeito foi estudada uma população de 85 profissionais dos Serviços Hospitalares de Anatomia Patológica, expostos ao FA no seu ambiente de trabalho e 87 indivíduos com historial ocupacional de não exposição ao FA e com características demográficas, de idade, sexo e hábitos tabágicos, semelhantes ao grupo exposto.

A avaliação da exposição ocupacional a FA foi realizada pela medição da concentração do FA no ar no local de trabalho e pela análise de diferentes indicadores biológicos. Para estimar o nível de exposição do FA nos Serviços de Anatomia recorreu-se à amostragem contínua de ar no posto de trabalho, representativa do ar inalado pelos trabalhadores. Os níveis de ácido fórmico na urina foram quantificados, por forma a estudar a sua potencial aplicação como biomarcador de dose interna de exposição ocupacional a FA. O estudo do dano genético foi avaliado em linfócitos do sangue periférico através de diversos biomarcadores de efeito, nomeadamente, alterações citogenéticas (frequência de aberrações cromossómicas, AC; micronúcleos, MN; e trocas entre cromatídeos irmãos, SCE), dano a nível do ADN, mediante o teste do cometa, e o teste de mutação do recetor das células T (TCR). A frequência de MN em células da mucosa bucal foi também avaliada. As possíveis alterações no sistema imunitário foram estudadas pela análise das percentagens das diferentes subpopulações linfocitárias. A fim de detetar diferenças genéticas responsáveis por variações na suscetibilidade dos indivíduos à exposição a FA, foram investigados polimorfismos de genes relacionados com o metabolismo (*CYP2E1, GSTM1, GSTT1, GSTP1*) e polimorfismos envolvidos no mecanismo de reparação de lesão do ADN (*FANCA, RAD51, XRCC2, XRCC3, XRCC1, PARP1, MUTYH, BRIP1*).

Para o grupo de trabalhadores estudados, tendo em conta a jornada de trabalho obteve-se um valor médio de exposição ao FA de 0.38 ± 0.03 ppm. O grupo exposto apresentou em média valores urinários de ácido fórmico estatisticamente superiores ao grupo controlo, no entanto não foi observada associação com os níveis de exposição no ar. Os resultados obtidos para os indicadores biológicos de genotoxicidade revelaram que os indivíduos expostos apresentaram, em média, maior dano genético comparativamente aos indivíduos do grupo controlo. Foi observado no grupo exposto um aumento significativo na frequência de AC, de SCE e no dano do ADN avaliado pelo teste do cometa. Observou-se ainda, nos trabalhadores, um aumento significativo na frequência de MN em linfócitos e em células da mucosa bucal. Foram encontradas associações (positivas) estatisticamente significativas entre a frequência de MN em ambos os tecidos, a duração (MN em linfócitos) e os níveis de exposição a FA (MN bucais), confirmando a sensibilidade deste biomarcador para avaliar o efeito genotóxico do FA em indivíduos ocupacionalmente expostos. Os profissionais dos serviços de Anatomia Patológica apresentaram também alterações nas percentagens de subpopulações linfocitárias, designadamente em células T citotóxicas, células *natural killer* e linfócitos B, comparativamente com o grupo controlo. No que concerne ao efeito dos polimorfismos genéticos na frequência dos diferentes biomarcadores, observou-se um aumento significativo do dano genético associado aos polimorfismos estudados nos genes

CYP2E1, *GSTM1* e *GSTT1* que codificam enzimas envolvidas na metabolização de xenobióticos. O mesmo efeito foi observado num dos genes que integram o sistema de reparação FANC, *FANCA*, revelando assim o envolvimento deste mecanismo na reparação da lesão genética induzida pela exposição ocupacional a FA.

No presente estudo constatou-se que os profissionais dos Serviços de Anatomia Patológica estão expostos a níveis de FA superiores a valores recomendados. Foi também observado um aumento significativo na frequência de biomarcadores de dano genético comparativamente com o grupo controlo. Face ao exposto, torna-se necessário tomar medidas de controlo. A primeira abordagem no controlo da exposição a um produto químico perigoso é analisar a possibilidade da sua substituição. Na sua impossibilidade, devem adotar-se medidas que reduzam ao mínimo o risco de exposição profissional, nomeadamente a implementação de boas práticas de trabalho e a formação dos trabalhadores nesses princípios. Os trabalhadores deverão também ser informados sobre a correta utilização dos meios de prevenção (coletiva e individual) e sua manutenção, de modo a minimizar os riscos inerentes às suas atividades. Paralelamente deverá proceder-se a melhorias nos locais de trabalho nomeadamente no que diz respeito às condições de exaustão e ventilação. Deve ser também assegurado um programa de vigilância da saúde dos trabalhadores que permita uma avaliação contínua dos riscos na saúde de natureza profissional e subsidie medidas e ações preventivas adequadas ao controle da exposição.

PREAMBLE

The modern way of life, along with population growth, is sustained by an industrial production never before attained, in which chemicals have become essential elements for the well-being and functioning of contemporary societies. Global production has increased over the time for 1 million tonnes in 1930 to 400 million tonnes today. Nonetheless, in spite of the value and usefulness of chemical products, their production, use and disposal can be harmful to human health and the environment. An important aspect of public health protection is the prevention or reduction of exposures to environmental agents that contribute, either directly or indirectly, to increased rates of premature death, disease, discomfort or disability. This assumes particular importance in occupational settings where the exposure to potential hazardous agents (in intensity, frequency and duration) is more significant. Chemical substances constitute the more extensive group of occupational risk factors, contributing significantly to the morbidity of the working population. The number of hazardous substances to which workers are exposed grows on a daily basis, reaching more than 100 000 in nowadays figures. For the most, there is little or none information about toxicity and potential health effects.

Measurement or estimation of actual worker exposure, along with appropriate evaluation of biologic parameters and assumptions about health effects or safety limits, is the standard method used to determine whether intervention is necessary and which forms of intervention will be more effective. During recent years, measures and strategies designed to prevent, control, reduce or eliminate occupational hazards and risks have been developed and applied, yet, despite continuous if slow improvements, work related diseases are still frequent (Alli, 2008) and in some cases, such as cancer, are rising (Clapp, 2008) thus their cost in terms of human suffering and economic burden continues to be significant.

In a recent survey conducted by the Portuguese Toxicology Association (ApTox) on the emerging issues in Environmental Health (EH) in Portugal, chemical exposure was identified by EH professionals as one of the principle hazards that currently contribute to most health problems in occupational settings. It was further predicted that in the next 5 to 10 years exposure to airborne chemicals will be the primary source of concern. Furthermore, during this period, the health points of concern are most likely to be respiratory and neoplastic disorders.

Formaldehyde is a high-volume production chemical produced worldwide with a large range of industrial and medical applications. It was recently reclassified as a human

carcinogen, based on increased evidence of cancer risk among subjects exposed to high measures (exposure level or duration) of airborne formaldehyde in the workplace. Several studies point the pathology and anatomy laboratories as one of the occupational settings where workers are more exposed to this aldehyde. Although formaldehyde genotoxicity and carcinogenic potentials are well documented in mammalian cells and in rodents, genotoxic effects and carcinogenic properties in humans are still conflicting and need more investigation.

The present study addresses the need for a policy-oriented research focused on the development of integrated approaches regarding the health risk assessment of populations occupationally exposed to hazard compounds. The prime objective is to provide rigorous information on the potential genotoxic effects of formaldehyde occupational exposure, towards a better understanding of the mechanisms of genotoxicity that may contribute to formaldehyde carcinogenicity in workers.

I. Review of the Literature

1. **INTRODUCTION**

"Preventing cancer is possible when we act on what we know."
(Clapp et al., 2008)

Humans are daily exposed to a variety of potentially harmful agents in the air they breathe, the liquids they drink, the food they eat and products they use (IPCS, 2002a). Long-standing evidence of the bond between health and the environment has led to the recognition of the need for sustainable development. On the other hand, there is an increasing global awareness of the inevitable limits of individual health care and the need to complement such services with effective public health strategies.

According to the World Health Organisation (WHO), cancer is a leading cause of death worldwide (WHO, 2013). Additionally, at least 200,000 people die every year from occupational or work-related cancers (WHO, 2007).

Most individuals spend one-third of their life in the workplace. Workers are often in contact with carcinogenic agents at higher levels than the general population, resulting in accumulation of exposure effects over lifetime. Many occupational diseases, including work-related cancers, are characterised by long latency periods and therefore are difficult to recognise until the clinical manifestation of their symptoms. Early detection of a professional risk situation can significantly decrease the occurrence of deleterious effects on worker's health. Thus, preventing individual exposure to carcinogens may prevent the disease.

Indeed, estimations show that at least one–third of all cancer cases of environmental and occupational origin can be prevented based on current knowledge (Espina *et al.*, 2013). This is best exemplified by the decline in lung cancer cases in males from the reduction in tobacco smoking, or by the drop in bladder cancer among dye workers from the elimination of exposure to specific aromatic amines (Clapp *et al.*, 2008; Jemal *et al.*, 2011).

Exposure assessment aims at prevention. Establishing the health effects of various activities and exposures requires information about the levels of exposure and the biological effects resulting from the interaction between the organism and the chemical agent. The resulting data provides a basis for designing effective prevention and mitigation strategies.

The current global labour force stands at about 45% of the world's population. Their work sustains the economic and material basis of society which is critically dependent on their working capacity. Thus health at work and healthy work environments are among the most valuable assets of individuals, communities and countries, and are critical components for sustainable development.

1.2 GENOTOXICITY AND CARCINOGENICITY

Genotoxicity is a comprehensive term that refers to the ability of an agent to damage DNA and/or cellular components regulating the fidelity of the genome and includes all adverse effects on genetic material (Eastmond *et al*., 2009). Genomic damage may be induced by several exogenous (exposure to genotoxic agents, lifestyle factors) and endogenous factors (genetic variants) (Holland *et al*., 2008). If the damage induced is not properly repaired it may give rise to genetic alterations that may ultimately lead to neoplasia and cancer (Rueff *et al*., 2002). Thus, genomic damage is probably the most important fundamental cause of developmental and degenerative disease.

Carcinogenesis is a multistep process that results from the accumulation of several genetic alterations controlling either cell death or cell proliferation leading to a progressive transformation of normal cells into highly malignant derivatives (Hanahan and Weinberg, 2000). Cancer is characterised by karyotypic instability, aberrant gene expression and uncontrolled cellular proliferation. Neoplasias may arise either from inherited mutations or from *de novo* mutations in somatic tissue (Jefford and Irminger-Finger, 2006). However, they are not only a consequence of DNA sequence mutations, but also from changes in the activities of genes and chromosomal regions (epimutations[1]) (Banno *et al*., 2012).

Human carcinogenesis is the outcome of a complex series of interactions between exogenous and endogenous processes (Kyrtopoulos, 2006). Although risk factors such as tobacco use, genetic predisposition, alcohol consumption, unhealthy diet, among others, play a major role in carcinogenesis process, a range of environmental variables and occupational exposures also contribute significantly to the global cancer burden (Clapp *et al*., 2008; Espina *et al.*, 2013). Indeed, over the last years, several epidemiological studies have revealed an increased risk of cancer development at various sites among subjects exposed to chemicals namely in occupational settings.

[1] abnormal transcriptional repression in active genes and/or abnormal activation of usually repressed genes (Banno *et al.*, 2012).

1.3 HUMAN EXPOSURE ASSESSMENT

Exposure assessment is a crucial procedure for the identification, evaluation and control of workplace hazards.

Human monitoring is a frequently used approach to provide early warning signals of excessive exposure to toxic substances and for prediction of health risks. Overall, it consists on a routine evaluation and interpretation of environmental and/or biological parameters, in order to detect possible health hazards before the poisoning or illness occur. In addition, it may contribute to define exposure limits for minimising the likelihood of significant health risks (Teixeira, 2004).

The exposure can be assessed by measuring the concentration of the chemical agent in environmental samples, such as air (Environmental Monitoring) and/or by measuring biological endpoints (Biological monitoring), called biomarkers or biological indicators. Generally, these two methods are used simultaneously, since they provide different but complementary information (IPCS, 2002a).

Environmental monitoring is the measurement of agents present at the workplace to evaluate ambient exposure and health hazard compared to an appropriate reference value. It quantifies or estimates the level of the chemical pollutant to which a worker is exposed during a task and/or workday. However, it represents only a snapshot of exposure in time and may not adequately reflect fluctuations in exposures. Furthermore, external exposure assessment does not take into consideration personal working habits or individual characteristics that may influence the uptake of the chemical agent.

Biological monitoring is any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological (IPCS, 1993). It provides an integrated measure of the level of exposure to chemicals through different pathways and exposure routes. Hence, it is essential not only for evaluating hazards to human health but also for developing strategies to improve occupational health and safety conditions and to assess the efficiency of the implemented policies.

The aim of human biomonitoring studies is to assess the risk of deleterious health effects by analysing the relationship between internal exposure and the biological effects in target cells. Selection of the optimal assay and tissue for analysis for any particular exposure situation should be based, whenever possible, on exposure type and duration, exposed population characteristics, endpoint mechanism, and the expected target tissue. Most of the time surrogate tissues (e.g. lymphocytes) rather than target tissues are studied for practical, methodological and ethical reasons (Albertini *et al*., 2000).

Biological monitoring encompasses the use of biomarkers as indicators to signal events of exposure to potential carcinogenic chemicals. Biomarkers are valuable tools in human biomonitoring studies, because they are intermediates between exposure and clinical manifestation of the disease, representing the whole continuum from external exposure to effect. It should be added that the detection of a biomarker not necessarily indicate the presence of a disease or toxic process, it may only indicate exposure of the organism to a substance (Juberg, 1999).

Biomarkers are traditionally classified into three types, biomarker of exposure, effect and susceptibility.

Biomarker of exposure- is the xenobiotic chemical itself, its metabolites or the product of an interaction between a chemical and its target molecule or cell that is measured in a compartment within an organism (IPCS, 1993). Generally, their presence only indicates that an exposure has occurred. A biomarker of exposure can be defined as a *biomarker of internal dose*, when the amount of a chemical or its metabolites is quantified in a biologic tissue or fluid (e.g. lead in blood) or as a *biomarker of biologically effective dose*, when it concerns the interactions between the chemical or its metabolites with target cellular macromolecules forming measurable covalent complexes (e.g. DNA-adducts or protein-adducts) (Teixeira, 2004).

Biomarker of effect- is defined as a measurable biochemical, physiological, behavioural, functional or other alteration within an organism that might be elicited by the exposure and depending upon the magnitude can be recognized as associated with an established or possible health impairment or disease (IPCS, 1993). It may represent an event that can be correlated with, and possibly predictive of, a deleterious health effect (NRC, 2006). These biomarkers are extensively used tools in occupational studies to evaluate the genotoxic potential of hazardous chemicals. Cytogenetic alterations, such as chromosomal aberrations and micronuclei are the most used endpoints. Moreover, it is well-established that chromosome aberrations in human lymphocytes predict a risk of cancer (Hagmar *et al*., 1998) the same association was recently confirmed for micronuclei in lymphocytes (Bonassi *et al*., 2007). Furthermore, the increased frequency of these indicators in exposed workers is a quite consistent finding in most studies (Suspiro and Prista, 2011), including in cancer-patients (Hagmar *et al*., 1998). The measurements of biomarkers of effect generally reflect events that take place at the latter end of the continuum between exposure and disease manifestation. Effect biomarkers, such as chromosomal aberrations or somatic mutations are not chemical specific, and therefore the association with an exposure must be established by an independent measure, such as environmental monitoring or simultaneous measurement of a biomarker of exposure.

Although they are less specific in identifying a single causative agent, they can be more predictive of ultimate toxicity. However, this will also depend on the individual variation in response to the exposure (Lutrell *et al*., 2008).

Biomarker of susceptibility- is an indicator of an inherent or acquired limitation of an organism to respond to the challenge of exposure to a specific chemical agent(s) (IPCS, 1993). Generally, it is used to identify whether a person or groups of people are susceptible to damage caused by certain chemical or other toxicant. This class of biomarkers serves to describe individual variations in the relationship of biomarkers of exposure and biomarkers of effect. These variations may help explain why different individuals with similar experiences of environmental exposure sometimes produce markedly different levels of biomarkers of exposure and/or biomarkers of effect, which in turn may correlate with different severities of clinical symptoms. These differences may arise from genetic or non-genetic factors. Genetic factors include polymorphisms that may lead to different individual capacities to activate, detoxify or repair DNA lesions arising from exposure to a chemical. Non-genetic indicators include among others, nutritional status, health status, lifestyle and age. These factors are independent of exposure but they identify those individuals in a population who may be more susceptible or resistant to the effects of a hazardous exposure.

Each of these biomarkers provides different information and consequently has its own set of advantages and disadvantages relating to its specificity, relevance to the toxic pathway, existence following exposure and difficulty of analysis (Teixeira, 2004). Ideally, it should be used various combinations of biomarkers or apply the same biomarker in different tissues or biological fluids in order to obtain more complex and comprehensive information and further expand the possibility to investigate the biological consequences of the exposure.

Given the above, estimation of the external exposure to an agent, the response of the organism to that agent and its potential susceptibility to toxic effects are all crucial parameters when assessing potential health effects associated to the exposure to an agent in the workplace. The basic tenets of biomarker-based environmental genotoxicity assessment are illustrated in Figure 1.

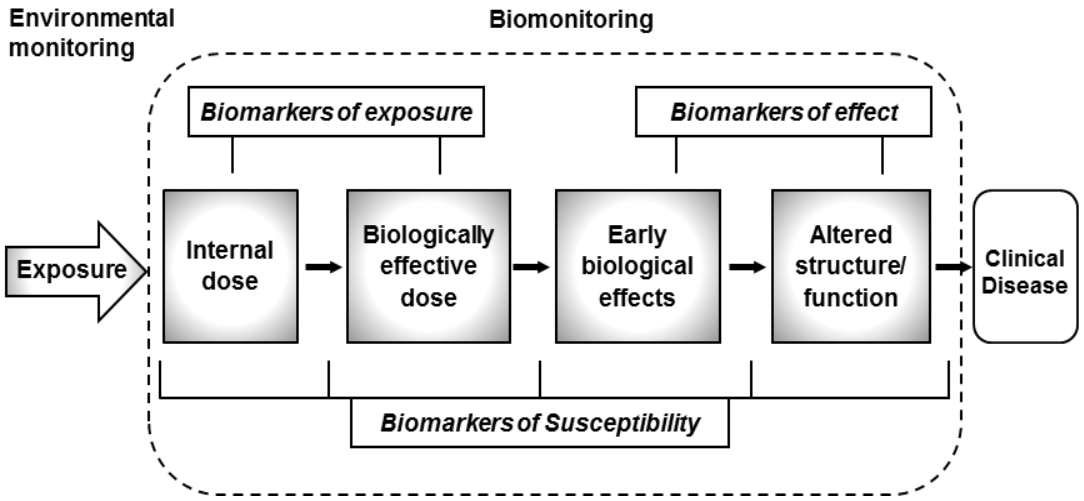


Figure 1. A simplified diagram of the cascade of events from exposure to clinical disease showing the relationships among exposure, effect and susceptibility biomarkers (*adapted from Manini et al. (2007)*)

2. **FORMALDEHYDE**

"You can run, but you can't formalde(hide)"

In a carton by Mike Baldwin

Formaldehyde was first synthesised by Aleksandr Butlerov in 1859, but was only in 1868 that it was conclusively identified by August von Hofmann, as the product obtained from passing a mixture of air and methanol over a heated platinum spiral. In fact, this production method laid the foundation for the modern manufacturing process of formaldehyde, via methanol oxidation using a metal catalyst (copper, molybdenum alloy, platinum or silver). At the end of the 19th century, formaldehyde was already used as a regular medical preservative and embalming agent. By the early 20th century driven by science and technology development, Leo Baekeland used a phenolformaldehyde resin to invent the first synthetic polymeric material, a hard moldable plastic, precursor of modern synthetic plastics. In 1940s, the first commercial particleboard was produced and a revolution was launched in the furniture and construction industries. Commercial applications continue to grow and today formaldehyde has an important role in both medical laboratory procedures and in the production of domestic and industrial goods that have become decisive in everyday life.

2.1 PHYSICAL AND CHEMICAL PROPERTIES

Formaldehyde (FA) is the most simple of all aliphatic aldehydes. It is a low-molecular weight organic compound, highly reactive, that readily combines with many chemical substances. At room temperature, FA is a flammable and colourless gas with a strong and distinctive odour. It is also known as methanal, methylaldehyde or oxymethylene.

FA is soluble in water, alcohols, and other polar solvents, but has a low degree of solubility in non-polar fluids. In dilute aqueous solution, the predominant form of FA is its monomeric hydrate, methylene glycol (or methanediol). Since it is rapidly polymerised at room temperature, methanol and other substances (e.g. amine derivatives) are frequently added as stabilisers to reduce intrinsic polymerisation. FA chemical structure is shown in Figure 2, its physical and chemical properties are listed in Table I.

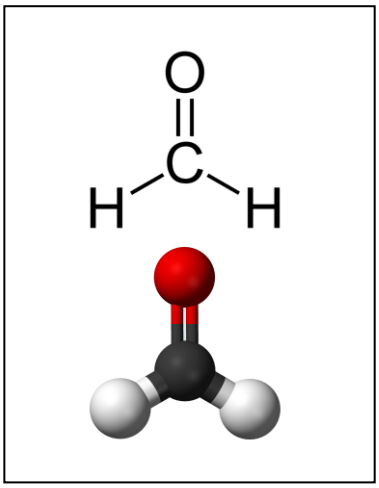


Figure 2. Formaldehyde 2D and 3D chemical structure.

2.2 ECONOMIC IMPORTANCE

2.2.1 Production

FA is a building-block of numerous compounds that are used in a wide array of materials and products. Due to its versatile properties and inexpensive production, FA is an economically important chemical produced worldwide. Generally FA is produced near the point of consumption because while it is easy to make, is costly to transport and can develop stability-associated problems during transportation and storage. As a result, international trade is minimal (NTP, 2011) and FA demand is provided by domestic industry.

FA is a high-volume production chemical with a global annual production and consumption of approximately 29 million tonnes (IHS, 2012). The European Union (EU) is the second largest producer, after Asia, producing over 7 million tonnes of FA each year, which accounts for about 25% of global production. China is the largest asian market, accounting alone for 34% of the world demand. The third larger market is the United States (U.S.), with an annual production of about 5 million tonnes (NTP, 2011). In fact, FA and FA-related products are allegedly responsible for more than 5% of the annual U.S Gross National Product (Zhang *et al*., 2009).

Moreover, it is forecast that the world consumption of FA will continue to grow, reaching an average rate of almost 5% per year throughout 2011–2016, especially due to increasing demand of fast growing economy countries industry (IHS, 2012).

2.2.2 **Commercial uses and applications**

FA is the most commercially important aldehyde, usually marketed as a 37 % (by weight) aqueous solution named formalin. Formalin also contains methanol (up to 15%) and other substances (several hundred milligrams per litre) that act as stabilisers. FA is also sold, to a lesser extent, in its solid form, either as a polymer, paraformaldehyde, or as a cyclic trimer, 1,3,5-trioxane.

The main industrial application of FA is in the manufacture of resins, namely, phenol-, urea-, melamine- and polyacetal resins.

Phenolformaldehyde and urea-formaldehyde resins are extensively used in furniture and construction industry as binders or adhesives for wood composites (sterling board, plywood, particleboard, fiberboard, hardwood plywood). Other applications include structural and acoustical insulation, foundry materials and heat-resistant components for automotive and aerospace applications and housewares. In addition, urea-formaldehyde resins are used in glass fiber roofing mats and paints. Melamine-formaldehyde resins are the raw material of several decorative applications including furniture, tableware, flooring, acrylic clear coats and paper finishing. The polyacetal resins are the most diversified with applications ranging from knife handlers, zippers, insulin pens, guitar picks and Bagpipes to automotive and consumer electronics. The leather, textile, rubber and cement industries are other examples where FA-based resins are widely used.

Another major application of FA is as an intermediate in the synthesis of other industrial chemicals including numerous organic compounds (e.g. 1,4-butanediol, pentaerythritol) and derivative substances, that in turn, will be used to make other products downstream (e.g. dyes, perfumes, vitamins).

FA is also used in agriculture, in controlled-release nitrogen fertilisers, crop protection agents and as a preservative in animal feeds. In a recent resolution, EU has decided to ban from the market FA-containing biocidal products for use as feed preservatives, from July 2015 (EU, 2013).

This aldehyde can be further applied as an anti-corrosive agent in electroplating processes and in the development of photographic films.

FA is also used in cosmetic products as a preservative and antimicrobial agent, namely in shampoos, deodorants, lotions, toothpaste, lipstick, mouthwashes, nail polish and hair products. Although FA *per se* is now rarely used in cosmetics, the use of FA-releasing preservatives is common.

FA application further extends to the health sector where it is used: i) in vaccines (as an inactivating agent) and pharmaceutical products; ii) to disinfect and sterilised hospital wards and medical equipment; iii) to preserve and embalm biological specimens.

In many instances, because FA is a long-established material with versatile properties, few compounds can replace it without compromising quality, performance and cost, which hinders its substitution to both producers and users.

2.3 **HUMAN EXPOSURE**

Given its economic importance and widespread use, many people are exposed to FA both environmentally and occupationally. The major route of exposure to FA is inhalation (IARC, 2006). Although environmental exposure typically occurs at much lower levels than occupational exposure, people are exposed to FA at some concentration in their daily lives. FA is also a naturally occurring biological compound present in all cells, tissues and body fluids (IARC, 2006).

According to WHO (2000) the estimates for the daily intake of FA in ambient air can vary between 0.002 and 0.04 mg/day mean values. On the other hand, for indoor air the average intake levels can differ between 0.3 and 0.6 mg/day. However, indoor air concentrations can be substantially increased with tobacco smoking, rising up to 0.5 to 3.5 mg/day. WHO also predicts that the mean daily intake of FA from occupational exposure is up to 8 mg/day, being variable according to the work in question.

2.3.1 **Environmental**

FA is ubiquitous in the environment and has been detected in indoor and oudoor air, soil, food, surface water, groundwater, treated and bottled water (NTP, 2011).

FA is formed primarily by the combustion of organic materials and by a variety of natural and anthropogenic activities.

Combustion sources include automobiles and other internal combustion engines, power plants, waste incinerators, refineries, wood stoves, forest fires and tobacco smoking. FA is also formed in the early stages of residual plant decomposition in the soil.

Secondary formation of FA occurs in the atmosphere through the photochemical oxidation of natural and anthropogenic hydrocarbon pollutants present in the air. In some instances, given the diversity and abundance of FA precursors in urban air, secondary production may exceed direct air emissions (IPCS, 2002b).

For the general population the major sources of FA exposure include combustion sources, tobacco smoke, offgassing from numerous construction and home-furnishing products, and offgassing from consumer goods.

FA levels in indoor air are often higher by one order of magnitude or more than those outdoors (IARC, 2006). The most significant source of indoor contamination is the release from newly home furnishings, primarily from wood pressed products made with urea-formaldehyde resins. These emissions are able to cause FA-exposure related symptoms in residents or occupants especially irritation of the eyes and upper airways (Salthammer *et al*., 2010). The most known example has occurred in the US, involving hurricane victims and government-provided FEMA (Federal Emergency Management Agency) trailers. Numerous victims of Hurricanes Katrina and Rita suffered adverse health effects (respiratory and other symptoms) as a result of being housed in FEMA trailers containing levels of FA 2-fold higher than the reference level. The FA was emitted from the materials used to build the mobile homes (CDC, 2008).

The general population can also be exposed to FA from consumer goods containing FA and from ingestion of food and water (IARC, 2006). Some of the products that often contain FA-releasers are cosmetics, household cleaning agents, soaps, shampoos, paints and lacquers (IPCS, 2002b).

Food and water contain measureable concentrations of FA, but the significance of ingestion as a source of FA exposure is not considered relevant (IPCS, 2002b; NTP, 2011). In food FA can be present either naturally (e.g. fruit, vegetables, shellfish) or as result of inadvertent contamination. FA-hydrated form, methylene glycol, can be found in surface water or groundwater but usually it is biodegraded within 1 to 15 days (IPCS, 2002b). In treated and drinking water FA is formed primarily through oxidation of organic matter during ozonation or chlorination treatment (NTP, 2011), yet its concentration is usually less than 100 µg/L (IPCS, 2002b).

2.3.2 **Occupational**

The major source of exposure to airborne FA occurs in the work environment. Occupational exposure to FA is transversal to a wide variety of professions and industries.

In the early 1990s it was estimated that approximately 1 million workers in the EU (near 36 000 in Portugal) and 2 million in the US were exposed to FA (FIOH, 1999; NTP, 2011). Although no current data is available in the literature, given that FA-world demand has grown over the last decades, it is safe to speculate that the number of individuals occupationally exposed to FA is now higher.

In occupational settings, FA occurs mainly as a gas, hence the major route of exposure is inhalation. Nevertheless, workers may also be exposed through dermal contact (formalin solutions) or by inhalation of FA-particulates such as powdered resins or wood dust (NTP, 2011).

Given its economic importance and wide spread use, occupational exposure to FA involves not only individuals employed in the direct manufacture of FA and products containing it, but also those using the products. Overall, the exposure to FA occurs in three main work-related scenarios:

- production and use of formalin in chemical industry (e.g., synthesis of resins) and in medical laboratories as a preservative and disinfectant agent.
- release from FA-based resins in which it is present as a residue and/or through their hydrolysis and decomposition by heat, during the manufacture of wood products, textiles and plastics.
- the pyrolysis or combustion of organic matter, e.g. in engine exhaust gases or during firefighting.

Another workplace circumstance that in the last few years has raised some concern is the FA emission from hair straightening products during hair treatment in hairdresser salons; although as shown by Pierce *et al*. (2011) this appeared to be limited to some specific products.

The occupational groups considered to be more at risk, either by duration or level of exposure, are industrial workers from chemical industries and plywood factories, embalmers and pathology and anatomy laboratory workers (IARC, 2006).

According to IARC (2006) the highest exposures to this aldehyde were measured in the past in industrial settings (e.g. varnishing of furniture and wooden floors, garment industry). However, recent legislation (e.g. resins that release less FA) and improved ventilation have resulted in decreased levels of exposure in many industrial workplaces.

Exposures to levels of up to 3 parts per million (ppm) have been reported for embalmers and for hospital laboratory professionals, such as anatomy and pathology workers (IARC, 2006). A recent study assessing the airborne exposure to FA in an industrial unit and anatomy pathology laboratory reveal higher levels of FA in the lab compared to the industrial setting (Viegas *et al*., 2010).

Furthermore, numerous epidemiological studies indicate a significant association between cancer risk and occupational exposure to FA (Hauptmann *et al*., 2004; Zhang *et al*., 2009). In fact, a recent study reported that the estimated cancer risk of laboratory technicians exposed to FA was 20% higher than the general population (Pilidis *et al*., 2009).

2.4 **TOXICINETIC AND METABOLISM**

The toxicokinetic behavior of FA has been the subject of several studies in a number of mammal species, and although there may be quantitative differences between them, the fate of FA is qualitatively similar among species (IARC, 2006).

The major route of exposure to FA is inhalation. At room and body temperatures, FA hydrates rapidly and it is in equilibrium with its hydrated form methylene glycol (or methanediol) (NTP, 2011). Owing to its reactivity and solubility, FA is readily absorbed from the respiratory tract following inhalation. In obligate nose breathers, such as rodents, absorption and deposition occur primarily in the nasal passages. In humans, which are oronasal breathers, it is likely to occur primarily in the nasal passages and oral mucosa, but also in the trachea and bronchus (Kimbell *et al.*, 2001). Species-specific differences of FA uptake and consequent associated effects (such as cell proliferation or reaction with other macromolecules) are determined by nasal airflow (nasal anatomy, ventilation) and breathing patterns rather than metabolism of the parent compound (Herausgegeben *et al*., 2006).

After oral exposure, FA is rapidly absorbed from the gastrointestinal tracts. In contrast, dermal uptake following application appears to be very slight, however it is able to penetrate skin (IARC, 2006).

FA is present at low levels in most living organisms. Physiological amounts of FA are endogenously formed from serine, glycine, methionine and choline, and also by demethylation of *N*-, *O*- and *S*-methyl compounds (IARC, 2006).

Nevertheless, regardless of its origin, endogenous or exogenous, FA has the same metabolic pathway in the organism (Figure 3) (ATSDR, 1999; IARC, 2006).

After absorption, FA is rapidly metabolised by oxidation to formate. The detoxification process takes place in all cells and can involve different enzyme systems. The primary and most important enzyme for the metabolic inactivation of FA is the glutathione-dependent formaldehyde-dehydrogenase (FDH) (also known as alcohol dehydrogenase class 3[2]; Koivusalo *et al*., 1989). FDH is highly specific for the FA-glutathione adduct, *S*-hydroxymethylglutathione, formed spontaneously in the cytosol from FA and glutathione conjugation. Formation of FA-glutathione adduct efficiently counteracts the presence of free FA, a reaction that is determined by the fact that glutathione is an abundant molecule in the cell. FDH oxidises *S*-hydroxymethylglutathione to *S*-formylglutathione, in the presence of NAD. This intermediate is then metabolised by *S*-formylglutathione hydrolase to yield formate and reduced glutathione (IPCS, 1989).

The activities of FDH are two to three orders of magnitude lower than those of *S*-formylglutathione hydrolase and thus the FDH-catalysed step is rate-limiting (IARC, 2006).

A secondary pathway involves the two aldehyde dehydrogenases (ALDH), ALDH1A1 (in cytosol) and ALDH2 (in mitochondria). Both enzymes have affinity for free FA but their *Km* values are higher than the *Km* displayed by FDH for *S*-hydroxymethylglutathione (Just *et al*., 2011). Therefore, FDH is probably the predominant enzyme responsible for the oxidation of FA at physiologically relevant concentrations, while ALDH enzymes contribute increasingly when the concentrations of FA increase (Just *et al*., 2011). Furthermore, some studies showed that FDH activity do not increase in response to FA exposure (Casanova-Schmitz *et al*., 1984; Øvrebø *et al.*, 2002) thus no increase in metabolism occurs and non-metabolised FA is probably bound to macromolecules.

Other alternative metabolic pathways include, FA oxidation to formate by catalase, but only in the presence of hydrogen peroxide (H_2O_2); and FA reduction to methanol by a cytosolic alcohol dehydrogenase class 1 (ADH1) (IARC, 2006) (Figure 3).

Being normal components of intermediary metabolism, neither FA nor formate are stored to any significant extent in any tissue. Formate is either excreted in the urine, as formic acid, or oxidised to carbon dioxide and exhaled.

Moreover, both FA and formate can be further incorporated into other cellular molecules, entering the 1 carbon intermediary metabolic pool, which involves the folic acid metabolic pathway for synthesis of nucleic acids, amino acids, and macromolecules (ATDSR, 1999).

[2] the systematic name for the enzyme is formaldehyde: NAD+ oxidoreductase (IUBMB code EC 1.2.1.46). Other names for this enzyme are formaldehyde dehydrogenase, NAD-linked formaldehyde dehydrogenase and NAD-dependent formaldehyde dehydrogenase.

The kinetics of FA metabolism and the effects on its rate of various enzyme inhibitors have been studied (IARC, 2006). In isolated rat hepatocytes, depletion of glutathione or inhibition of the metabolic enzymes, FDH, ALDH2 or ADH1 were found to decrease FA metabolism and to increased cytotoxicity in a dose-dependent manner, through reactive oxygen species (ROS) production (Teng *et al*., 2001). Recent studies have identified ALDH2 as the primary enzyme responsible for the detoxication of aldehydes in haematopoietic stem cells, the lack of this enzyme results in hypersensitivity of haematopoietic stem cells to exogenous aldehydes, by accumulating DNA damage (Langevin *et al*., 2011; Garaycoechea *et al*., 2012). Thus, it is reasonable to assume that individuals with lower levels of glutathione as well as those with deficiencies in any of the above enzymes (by altered genes or polymorphisms) will be more susceptible to FA toxicity.

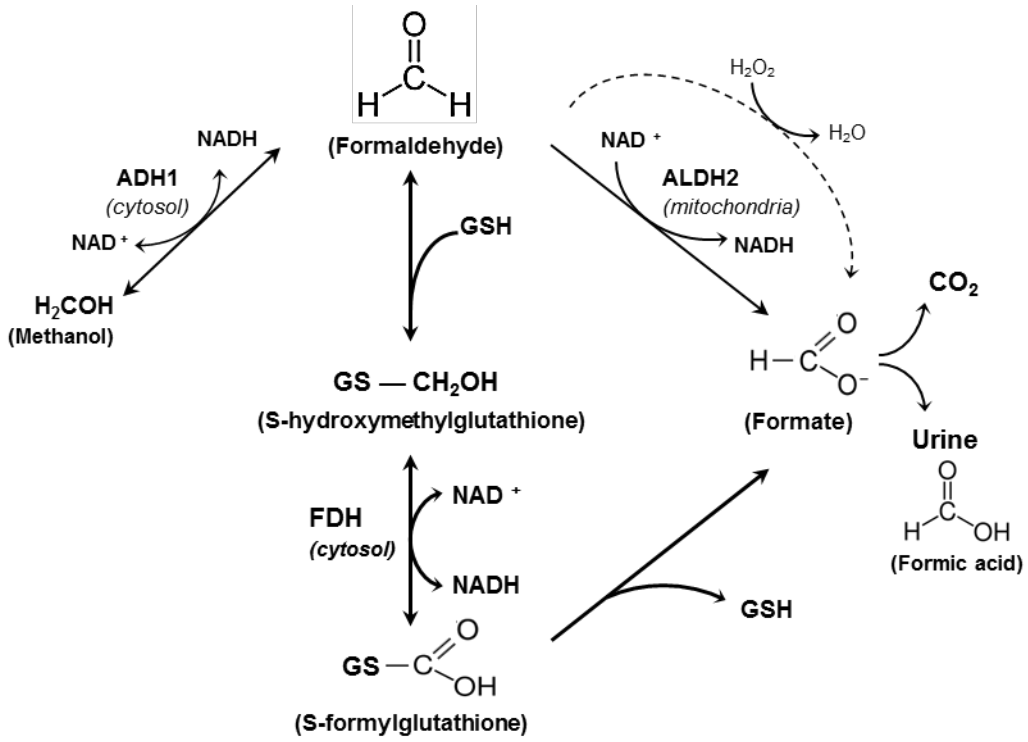


Figure 3. Fate and metabolism of formaldehyde (*adapted from Teng et al. (2001) and IARC (2006)*).

2.5 **HEALTH EFFECTS**

2.5.1 **Sensory irritation and related symptoms**

FA has a pungent and suffocating odour and at certain concentrations is a strong irritating gas (ATDSR, 2010).

The respiratory tract, especially the upper respiratory tract, is a critical target of the irritant action of inhaled-FA, as shown by human acute controlled exposure studies, by studies on humans exposed acutely or repeatedly under occupational or residential conditions, and by studies of animals exposed by inhalation for acute, intermediate, and chronic durations (IARC, 2006; ATDSR, 2010).

Most humans detect and/or recognise FA odour at concentrations below 1 ppm (ATDSR, 2010), but the odour threshold has been reported to be between 0.05 and 0.18 ppm (Arts *et al.*, 2006). The most common exposure effects to FA vapours comprise sensory irritation of the eyes, nose and throat, with eye irritation generally accepted as the most sensitive endpoint.

Odour perception and the threshold for sensory irritation are sometimes not clearly differentiated, it generally exist on a concentration gradient, but may overlap (Gaffney and Paustenbach, 2007). In FA case, however, the progression of responses is clear, with human studies showing that for most individuals, odour perception generally precedes sensory irritation (Golden, 2011). Importantly, odour perception and sensory irritation are different and distinct physiological processes. Upon substance inhalation odour is the sensation of smell carried by the olfactory nerve (first cranial nerve), while sensory irritation involves stimulation of the trigeminal nerve endings, and near triggering concentrations is generally considered to be a physiological and not a toxic response (Gaffney and Paustenbach, 2007; Golden, 2011).

Sensory irritation encompasses a graded series of responses related to the concentration of FA in the air and is generally categorised as ranging from slight to moderate (mucosa irritation) to severe (e.g. difficulty in breathing). The response also depends on the length of exposure and individual sensitivity.

The most common symptoms, irritation of the eyes, nose, and throat, along with increased tearing, occurs at FA-concentrations of about 0.3 - 3 ppm in the air.

Other symptoms described by individuals exposed to FA include sneezing, coughing, sinus, sore throat and nausea (Lang *et al.*, 2008). In fact, clinical symptoms of eye soreness (68%), lacrimation (60%), eye fatigue (45%), rhinorrhea (38%), and throat irritation (43%) were reported in a survey involving 143 medical students exposed to a mean FA concentration of 2.4 ppm, during a course (15 hours/week for 2 months) (Takahashi *et al*., 2007). Similar symptoms were also described by students but at a lower airborne FA concentration, 0.72 ppm (6-8 hours/day for 3 months) (Wei *et al.*, 2007).

Neurological effects due to FA exposure were also reported by a few studies namely, decrease performance in short-term memory tests and in the ability to concentrate (Bach

et al., 1990; ATDSR, 2010). Indeed, in animal studies impaired learning/memory and altered motor activity were reported following acute- and intermediate-duration exposure to FA (ATDSR, 2010).

Changes in human pulmonary function, detected by spirometry tests (FEV, FVC), are assumed to occur at 0.6 ppm (ATDSR, 2010), but probably as protective response of the organism to the exposure, since the exposure to sensory irritants of the upper respiratory tract often cause slower breathing or even breath holding (Gaffney and Paustenbach, 2007). Volunteers (healthy and asthmatic) in acute controlled exposure studies showed no significant changes in pulmonary function after exposure to FA. In chronic exposures, some authors reported a decrease in pulmonary function of workers exposed to FA (Akbar-Khanzadeh *et al*., 1994; Fransman *et al*., 2003; Tang *et al*., 2009), while others found no exposure-related differences (Arts *et al*., 2006; ATDSR, 2010).

Increased numbers of eosinophils and protein exudation following FA exposure (0.4 ppm) were found in nasal lavage studies confirming the irritative effect of FA; a non-specific pro-inflammatory effect of FA was suggested by the authors (Pazdrak *et al.*, 1993).

Nasal epithelial lesions have been observed consistently across a few occupational studies (ATDSR, 2010). In addition, clinical findings of upper respiratory tract inflammation were reported in 41% of workers exposed to FA (0.71 ppm) namely, hypertrophy or atrophy of the upper respiratory mucous membranes, chronic pharyngitis, rhinitis, rhinosinusitis, and rhinopharyngitis (Lyapina *et al*., 2004).

2.5.2 **Sensitisation**

FA (vapour) is considered an etiologic factor in occupational asthma, but although it may cause asthma in some individuals, this occurs rarely (Ezratty *et al*., 2007) and it cannot be regarded as a potent asthmogenic agent (Kim *et al*., 2001).

In fact, over the years, the association between FA-exposure and asthma (or exacerbation of) has been controversial, with investigations providing limited evidence of a causal relationship.

Nevertheless, a few case-reports of asthma associated to occupational exposure to FA were confirmed by bronchial provocation tests (Kim *et al*., 2001; IARC, 2006; Tang *et al*., 2009). The mechanism of FA-induced asthma remains unclear (Kim *et al*., 2001). However, it seems to be related to a delayed-type hypersensitivity reaction, since the subjects showed a latent period of symptomless reaction after exposure (IARC, 2006).

A review on controlled chamber studies involving asthmatic volunteers found that acute exposures to FA up to 3 ppm are unlikely to provoke asthma in an unsensitised asthmatic (Liteplo and Meek, 2003), yet high levels of FA may probably cause asthmatic reactions by an irritant mechanism (IARC, 2006).

Interestingly, a non-occupational study reported a significant relationship between asthma in children and residential indoor concentration of FA, at low levels (above 0.05 ppm) (Rumchev *et al*., 2002). This finding was recently explored by a meta-analysis of seven peer reviewed studies which confirmed the positive association between indoor inhalation of FA and induction/exacerbation of asthma in children (McGwin *et al*., 2010). Some authors however stated that these reports were confounded by potential co-exposures and other factors (Arts *et al*., 2006; Golden, 2011). Among adults, a few studies have reported a positive association between indoor levels of FA and asthma while others have not (McGwin *et al*., 2010). The effects of FA-indoor levels appear to be less evident in adults than in children (especially in asthmatics) living in the same household (Krzyzanowski *et al.*, 1990).

The question whether FA vapour exposure is a risk factor for occupational or childhood asthma (or its exacerbation), or not, or may act in concert with other identified or as yet unidentified factors, still remains unanswered (Golden, 2011). Further data from other studies suggested a possible association between exposure to FA and allergic sensitisation to common aeroallergens in children and in asthmatic subjects (Garrett *et al*., 1999; Casset *et al*., 2006) however a recent study found no significant deleterious effect on airway allergen responsiveness of subjects with intermittent asthma (Ezratty *et al*., 2007).

FA (formalin solution) is a known skin sensitizer and one of the common causes of allergic contact dermatitis (IARC, 2006). Skin sensitivity to FA has been associated with many situations of dermal exposure, including contact with formalin, FA-containing resins, FA-treated fabrics and FA containing household products (Kim *et al*., 2001). Furthermore, FA has been widely reported to cause dermal allergic reactions in occupationally exposed workers, namely, laboratory workers, hairdressers, textile workers and industry workers (Kim *et al*., 2001; Aalto-Korte, 2008).

2.5.3 **Genotoxicity**

In the last two decades, a large number of studies were published on the genotoxicity of FA in a variety of biological systems and endpoints. The vast majority demonstrate FA

genotoxicity in a multiple of experimental *in vitro* and *in vivo* models and in exposed humans.

In fact, FA has given positive results for almost all genetic endpoints evaluated in bacteria, non-mammalian eukaryotes (yeast, fungi, plants, insects, nematodes), *in vitro* studies with proliferating mammalian cell lines, and *in vivo* animal studies (IARC, 2006; NTP, 2011).

The evidence of FA-induced mutations *in vitro* experimental systems is consistent and generally associated with clastogenic effects and direct DNA mutation.

FA showed mutagenic potential in *Salmonella typhimurium* and *Escherichia coli*, with or without metabolic activation, including forward and reverse mutations (Schmid *et al*., 1986; IARC, 2006) and microsatellite instability (Wang *et al*., 2007).

Studies in non-mammalian eukaryotes and plants were also positive for forward and reverse mutations, lethal mutations and DNA single-strand breaks (IARC, 2006). For example, yeast strains deficient in repair pathways showed to be more susceptible to the mutagenic effects of FA (Magaña-Schwencke and Ekert, 1978).

Similarly, *in vitro* treatment of mammalian cells with different concentrations of FA induced several genotoxic endpoints including, deletions, point mutations, insertions, cell transformation, cytogenetic alterations (chromosomal aberrations, sister chromatid exchanges and micronucleus), DNA-protein crosslinks, DNA single-strand breaks and DNA- adducts (IARC, 2006).

In fact, chromosomal aberrations (CAs), sister chromatid exchanges (SCEs) and micronucleus (MN) were all increased *in vitro* in several human and rodent primary cells and cell lines (IARC, 2006, 2012). Schmid and Speit (2007) observed elevated MN formation in human lymphocytes isolated from whole blood cultures after *in vitro* exposure to FA (at 44h). Further evidence of FA-induced MN was found in DNA-repair-deficient cell lines (Ridpath *et al*., 2007), indicating the importance of certain repair pathways in the prevention of DNA lesions caused by FA exposure. A549 human lung cells and V79 Chinese hamster cells showed increased frequency of SCEs after treatment with different concentrations of FA. One hour after the addition of the agent to the A549 cells, the culture medium still retained the capacity to produce SCEs in non-exposed V79 cells, suggesting that genotoxicity persists despite the high reactivity of FA with macromolecules in the culture medium (Neuss and Speit, 2008; IARC, 2012).

Other *in vitro* studies described the formation of DNA-protein crosslinks in mammalian cells treated with FA (Conaway *et al*., 1996). Some of the proteins enrolled in these lesions were identified in cellular functions, namely transcription, gene regulation, DNA replication, and DNA repair (Qiu and Wang 2009).

It has also been reported that FA is able to induce hydroxymethyl adducts in mammalian DNA. For instance, DNA adducts were formed in an experimental reaction with FA and deoxyribonucleosides (Cheng *et al.*, 2008a), DNA from human placenta (Zhong and Hee 2004) and DNA from human nasal epithelial cells (Speit *et al*., 2008). In addition, Lu *et al*. (2009) demonstrated the formation *in vitro* of an adduct between DNA and *S*-hydroxymethylglutathione, a key intermediate in cellular FA detoxication (please see 2.4).

Laboratory animals inhaling FA have generally shown more genotoxic effects in the nasal tissues. In rats FA vapour inhalation resulted in CAs in pulmonary lavage cells (Dallas *et al*., 1992), MN induction in the gastrointestinal tract (Migliore *et al*., 1989), DNA-protein crosslinks in the nasal mucosa and fetal liver (IARC, 2006), DNA strand breaks in lymphocytes and liver (Im *et al*., 2006; Wang and Liu, 2006) and dominant lethal mutations (Odeigah PG, 1997). However, no significant increase in CAs, MN or SCE frequencies were found in peripheral blood of rats (IARC, 2006, 2012).

A number of human biomonitoring studies have examined the genotoxic effect of FA in occupationally exposed populations, with both positive and negative outcomes. A wide variety of genotoxic endpoints has been investigated, particularly cytogenetic alterations, DNA damage and more recently DNA adducts. A fundamental aspect in the assessment of genotoxic effects of FA in exposed humans is whether genotoxicity is limited to first contact tissues or not. Therefore, genotoxic evaluations had focused on local-site tissues such as nasal and buccal mucosa cells or in circulating blood lymphocytes, nevertheless, there are few studies published that combined these two type of tissues (Ye *et al*., 2005; Viegas *et al*., 2010; Costa *et al*., 2013).

Several studies have reported a significant increased frequency of MN (buccal, nasal and lymphocytes) (Suruda *et al*., 1993; He *et al*., 1998; Orsière *et al*., 2006), SCEs (He *et al*., 1998; Shaham *et al*., 1997, 2002), CAs (He *et al*., 1998, Jakab *et al*., 2010), DNA-protein crosslinks (Shaham *et al*., 1997, 2003) and DNA damage (by Comet assay) (Yu *et al*., 2005; Costa *et al*., 2008) in FA-exposed workers, namely, industry workers, embalmers and pathology and anatomy workers.

A few studies have identified and quantified FA adducts in occupational exposed individuals and in smokers (FA is one of the main components of tobacco). Pala *et al*. (2008) reported a significant increase in FA- serum albumin conjugates in a small group of FA- exposed laboratory workers compared to referents. Further, a recent study by Bono *et al*. (2010) found a significant increase on the frequency of leukocyte malondialdehyde-deoxyguanosine adducts (M1-dG), a biomarker of oxidative stress and lipid peroxidation in a group of FA-exposed pathologists compared to controls. In smokers (≥ 10

cigarettes/day), Wang *et al*. (2009a) has identified a specific FA-DNA adduct. The N^6-hydroxymethyldeoxyadenosine (N^6-HOMe-dAdo) adduct was detected in 29 of the 32 smokers and in 7 of the 30 non-smokers, the difference was statistically significant. Furthermore, besides the tobacco smoke, authors proposed other minor sources of FA, namely as a by-product of another tobacco-specific compound or as result of lipid peroxidation or inflammation.

Also of note, a set of 89 miRNAs were significantly dysregulated in human lung cells treated with FA. Mapping of changed miRNAs and predicted mRNA targets revealed an association between FA exposure and inflammatory response pathways (Rager *et al*., 2011).

The formation of FA-DNA adducts in leukocytes of FA exposed subjects demonstrate that inhaled FA is able to enter systemic circulation and produce genotoxic effects in circulating cells, which may ultimately lead to carcinogenesis in distal sites.

Furthermore, it is believed that FA-genotoxicity and cytotoxicity have a key role in FA-related human carcinogenesis (NTP, 2011).

Genotoxic endpoints analysis, either cytogenetic alterations or DNA adducts, are of great interest in risk assessment of occupational carcinogens because they precede by a long time the potential health effects, thus offering a greater potential for preventive intervention (Mayeaux R., 2004).

2.5.4 **Carcinogenicity**

In 2004, the International Agency for Research on Cancer, IARC, reclassified FA from Group 2A, *probably carcinogenic to humans*, to Group 1, *carcinogenic to humans* (IARC, 2006). Recently, the US National Toxicology Programme (NTP) has revised FA carcinogenic status and after a rigorous scientific review, FA was listed in the NTP 12th Report on Carcinogens as *known to be a human carcinogen* (NTP, 2011). Both reclassifications are based on sufficient epidemiological evidence from human studies, experimental evidence in animals and supporting studies on mechanisms of carcinogenesis.

2.5.4.1 ***Epidemiological studies***

Numerous epidemiological studies have evaluated the relationship between cancer risk and exposure to FA in occupational settings, with focus on industry workers, embalmers and health professional groups namely physicians, pathologists and anatomists.

Epidemiological studies have demonstrated a causal relationship between exposure to FA and cancer in humans (NTP, 2011). Causality is indicated by consistent findings of increased risks of nasopharyngeal cancer and leukaemia, among individuals with higher measures (exposure level and duration) of exposure to FA, which cannot be explained by chance, bias or unrecognized confounding effects (NTP, 2011; IARC, 2012). In addition, a positive association was observed between exposure to FA and sinonasal cancer (IARC, 2012). A number of studies have also found associations between FA-exposure and cancer at other sites, including the oral cavity, oro- and hypopharynx, pancreas, larynx, lung and brain, however, the overall evidence does not support a causal role for FA in these cancers (NTP, 2011; IARC, 2012).

Some of the more important studies implicated in FA carcinogenicity in humans are briefly described below.

Nasopharyngeal cancer

The nasopharynx comprises the upper part of the throat that lies behind the nose. Studies have shown that all three histological types of nasopharyngeal cancer (undifferentiated carcinoma and keratinising and non-squamous cell carcinoma) start from the epithelial cells of the nasopharynx. The nasopharyngeal cancer is a rare cancer representing about 0.7% of the global cancer burden (Jemal *et al*., 2011).

The sufficient epidemiological evidence that FA causes nasopharyngeal cancer comes from five case–control studies and from the largest and most informative cohort study available to date involving workers from ten US industries (25 619 subjects), conducted by the National Cancer Institute (NCI).

In the NCI cohort study, a statistically significant excess of deaths from nasopharyngeal cancer was found in subjects occupationally exposed to FA in comparison with the general population (Hauptmann *et al*., 2004). Furthermore, a highly significant exposure–response correlation was observed between cancer mortality rate and peak (ppm) and cumulative exposures (ppm-years). Controlling for co-exposure to other potential occupational carcinogens and bias did not alter the exposure-response relationship. All cases were in the highest level of exposure category, most of which occurred in one plant, Plant 1.

A separate case-control study nested in NCI cohort was conducted to investigate if the excess mortality from nasopharyngeal cancer found in Plant 1 were related to other factors, namely, occupational history outside the FA industry. Marsh *et al*. (2007) found that silversmithing, prior to or after employment in Plant 1, was associated to

nasopharyngeal cancer risk and concluded that the observed excess of nasopharyngeal cancers may be due to other occupational exposures rather than FA exposure, e.g silversmithing. However, several authors observed that while there was some indication of influence by activities as a silversmith, confounding alone did not explain the relatively high number of deaths from nasopharyngeal cancer found in this plant (Nielson *et al*., 2010; IARC, 2012). Furthermore, in Hauptmann *et al*. (2004) risks related to duration, cumulative, and average intensity of FA exposure did not decrease when silversmithing or other metal work were added as adjustment variables, indicating that silversmithing did not confound the association between FA and nasopharyngeal cancer.

Recently, the NCI cohort study was extended by 10 years (through 2004) the results from this update continue to indicate a link between FA occupational exposure and nasopharyngeal cancer, consistent with previous findings (Beane *et al.*, 2013).

Increase incidences of deaths from nasopharyngeal cancer were also found in two other cohort studies: in a Danish study of proportionate cancer incidence, among subjects working in companies that manufactured or used FA (Hansen and Olsen, 1995) and in a proportionate mortality analysis of the largest US cohort of embalmers (Hayes *et al*., 1990).

Conversely, no excesses of nasopharyngeal cancer mortality was detect in other large cohort studies, two among industrial workers (Coggon *et al*., 2003; Pinkerton *et al*., 2004) and one among embalmers and funeral directors (Walrath and Fraumeni 1983). However, in these studies the number of cases were fewer than expected and the statistical power to detect an effect on nasopharyngeal cancer (rare cancer) was low (IARC, 2006).

Five of seven case-control studies showed consistent findings of increased risk of nasopharyngeal cancer among individuals with the highest or overall exposure to FA, but not all were statistically significant (Vaughan *et al.*, 1986, 2000, Roush *et al.*, 1987, West *et al.*, 1993, Hildesheim *et al*., 2001).

The studies by Vaughan *et al*. (2000) (multi-center) and Hildesheim *et al*. (2001) are regarded as the most informative because of their size, their exposure assessment and more importantly the evaluation of potential confounders (IARC, 2012). In both case-control studies, risks of nasopharyngeal cancers increased with exposure duration (years) and cumulative (ppm-years) exposure to FA.

Furthermore, Vaughan *et al*. (2000) stratified the analysis by histological subtype of nasopharyngeal cancer and used several different measures of exposure to evaluate risk. A statistically significant exposure-response trend was found for squamous and unspecified epithelial carcinomas. The risk of nasopharyngeal cancer, all types, was also

increased, but only for workers whose exposure duration were the longest, and not necessarily the highest.

On the other hand, Hildesheim *et al*. (2001) found some evidence of increasing risk of nasopharyngeal cancer with duration of exposure, but the observed trend did not reach statistical significance. However, a strong effect was observed for Epstein-Barr seropositive (EBV) individuals. In fact, a significant increase in risk of nasopharyngeal cancer was found among those ever-exposed to FA and EBV positive. It has been suggested that FA may have an indirect influence in EBV reactivation through deregulation of nitrosothiol homeostasis and in addition may also interact with the virus to promote epithelial cell transformation (Thompson and Grafström, 2009).

Overall, in the five case-control studies higher risks were found among FA-exposed individuals in the high-exposure groups (Vaughan *et al.*, 1986, Roush *et al.*, 1987) or with more years since first exposure (West *et al.*, 1993). Some studies also reported that the risk remained elevated after consideration of/or adjustment for tobacco smoking (West *et al.*, 1993; Vaughan *et al.*, 2000; Hildesheim *et al.*, 2001) or exposure to wood dust (Vaughan *et al*., 2000; Hildesheim *et al.,* 2001).

However, other case-control studies did not find an association between FA exposure and nasopharyngeal cancer (Armstrong *et al.,* 2000, Hauptmann *et al.*, 2009).

A few meta-analyses with some of the above studies, cohort and/or case-controls, are available in the literature, but with contrasting results. Bosetti *et al.* (2008) did not found a significant increase in nasopharyngeal cancer risk in three cohort mortality studies. On the other hand, Bachand *et al*. (2010) found a near significant risk in a meta-analysis of seven case-control studies, while for six cohort studies no significant increase was detected (after exclusion of Plant 1 data of NCI cohort).

Lymphohematopoietic cancer

An excess of mortality from leukaemia or combined lymphohematopoietic cancer (cancer of the lymphatic and blood-forming systems) has been consistently observed in epidemiological studies involving embalmers, funeral directors and health professionals, namely, pathologists and anatomists (IARC, 2006, 2012).

Several of these studies, however, only examined cancer outcomes by occupation and occupational characteristics (e.g., duration of employment) without exposure assessment. A recent nested case-control study by Hauptmann *et al*. (2009) examined exposure-response relationships for lymphohematopoietic malignancies among embalmers and funeral directors by metrics of exposure (average, cumulative, peak, and duration of

embalming) based on lifetime work histories, practices and predicted FA-exposure levels. Mortality from myeloid leukaemia was significantly elevated among ever-embalmers and significant trends were observed with duration and peak exposure to FA.

Three of the largest cohorts in industrial settings were updated, and two found evidence of an excess of deaths from leukaemia and lymphohematopoietic cancer associated with FA exposure, namely in the US NCI industrial cohort (Hauptmann *et al*., 2003; Beane *et al*., 2009) and in the US cohort of garment workers (Pinkerton *et al*., 2004). Conversely, no excess of mortality for leukaemia was found among workers exposed to FA in a British industrial cohort study (Coggon *et al*., 2003).

Overall, the above studies found positive exposure-response relationships for combined lymphohematopoietic cancer or specific subtypes, but the strongest association observed was for myeloid leukaemia (NTP, 2011). For instance, in Meyers *et al.* (2013) although myeloid leukaemia mortality was elevated in garment workers compared to the US population, the overall leukaemia mortality was not.

The large embalmers nested case-control study by Hauptmann *et al*. (2009) and the cohort studies updates of NCI industrial workers (Hauptmann *et al*., 2003; Beane *et al.*, 2009) are considered to be the most informative studies for myeloid risk evaluation related to FA exposure (NTP, 2011). In these studies, statistically significant exposure-response relationships were observed with peak, average exposure and duration of exposure to FA. Controlling for co-exposure to other potential occupational carcinogens did not alter the findings.

In Beane's *et al.,* (2009) follow-up study the overall risk of myeloid leukaemia although elevated was declined compared to previous reports. This finding is consistent with largest risks occurring closer in time to relevant exposure (Beane *et al*., 2009) and resembles the wavelike pattern of the relative risks over time seen for known leukaemogenic agents (e.g. benzene) (Beane *et al.,* 2009; NTP, 2011).

Few case control studies have assessed the relationship between FA-exposure and risk for leukaemia (Linos *et al*., 1990; Partanen *et al*., 1993; Blair *et al*., 2001). No clear association was found in any of the studies, probably due to the small number of exposed cases and limited statistical power to analyse exposure-response associations (Zhang *et al*., 2009).

Three meta-analysis were recently published with contrasting results. The meta-analysis by Zhang *et al.* (2009), which included 15 cohort and case control studies found a significantly elevated risk of leukaemia, specially, myeloid leukaemia. In opposition, a

meta-analysis by Bachand *et al.* (2010) did not find a significantly elevated risk for leukaemia or subtypes associated to FA-exposure in the studies selected for analysis.

Bosetti *et al.* (2008) found an elevated risk of leukaemia across studies of professional groups (pathologists, anatomists and embalmers) but not across studies of industrial workers. This finding is consistent with observations by Hauptmann *et al.* (2009) that embalmers have longer duration of FA-exposure, higher cumulative exposure and are more likely to be exposed to peak exposure levels greater than 4 ppm than industrial workers.

Sinonasal cancer

Sinonasal cancer is a rare disease with an annual incidence of about 1 per 100 000 and it comprise cancers of the nasal cavity and paranasal sinus (Luce *et al*., 2002).

A number of case–control studies have shown a positive association between sinonasal cancers and occupational exposure to FA (Olsen *et al.*, 1984, Olsen and Asnaes 1986, Roush *et al.*, 1987, Luce *et al.*, 1993). In most studies, the significant increase of sinonasal cancer risk was associated with FA exposure–response patterns, even among workers with low or no exposure to wood dust or after adjusting for exposure to wood (Olsen *et al*., 1984, Olsen and Asnaes 1986, Luce *et al*., 1993, 2002).

Elevated risks were found for the two major types of sinonasal cancer types, adenocarcinoma and squamous-cell carcinoma, but in some studies, the association was stronger for the adenocarcinoma (Luce *et al*., 1993, 2000; NTP, 2011).

The increased risk of adenocarcinoma among FA-exposed workers was confirmed in a pooled analysis study, which collected and re-analysed data from 12 case-control studies (Luce *et al*., 2002), furthermore the increased risk was followed by an increased cumulative exposure (ppm-years).

Other case-control studies did not find an association between FA exposure and sinonasal cancer (Vaughan *et al*., 1986; IARC, 2012) due probably to the small sample size (NTP, 2011).

Collins et al. (1997) meta-analysis found a significant association between FA-exposure and mortality from sinonasal cancers, nonetheless the author pointed out that the result was probably confounded by concomitant wood dust exposure detected in few of the studies included.

Mix results were found with regard to cohort studies. A statistically significant excess of sinonasal cancer was obtained in a Danish cohort study among workers exposed to FA

(Hansen and Olsen, 1995) and a non-significant excess of deaths from sinonasal cancer was found in the NCI cohort, among industry workers exposed to FA (Hauptmann *et al*., 2004).

No increase in deaths from sinonasal cancers was found in other large cohort studies, (industry workers, Coggon *et al*., 2003; garment workers, Pinkerton *et al*., 2004) nor in a meta-analyses of eight industrial cohort studies (Bosetti *et al*., 2008).

It should be noted that in these cohort studies the statistical power to detect an association between FA exposure and sinonasal cancer (rare cancer) was limited, and even the larger cohort studies had insufficient numbers of exposed workers and expected deaths (e.g. approximately three in the NCI cohort) (NTP, 2011).

Nonetheless, based on the data available the NTP commission found that there was sufficient epidemiological evidence that FA occupational exposure cause sinonasal cancer in humans (NTP, 2011). On the other hand, the IARC (2006, 2012) working group concluded that the existing studies only offered limited positive evidence of a causal relationship between FA exposure and sinonasal cancer (IARC, 2006). The major limitations identified were the concomitant exposure to wood-dust, strongly associated with sinonasal cancer in some case-control studies, and the discordant results between the cohort and case control studies, which may be due to lack of statistical power or could indicate the confounding effect of wood dust (IARC, 2012).

2.5.4.2 ***Animal studies***

FA exposure caused tumours in two rodent species (rats and mice) at several different tissue sites and by two different routes of exposure (inhalation and ingestion) (NTP, 2011).

First evidence of the carcinogenic potential of FA came from long-inhalation studies with rodents (IPCS, 1989). These studies demonstrate that high concentrations of FA can cause irreversible damage to the nasal epithelium of rats and that in some cases rats exposed continually to these concentrations developed neoplasia (Merck and Speit, 1998).

In five studies the long-term inhalation exposure to FA caused nasal tumours, both benign (polypoid adenoma) and malignant (predominantly squamous-cell carcinoma but also adenocarcinoma and carcinoma) in two rats strains: male and female F344 rats (Monticello *et al*., 1996, Kamata *et al.*,1997) and in male Sprague-Dawley rats (Sellakumar *et al*., 1985; IARC, 2006). Nasal lesions were also observed in male B6C3F1

mice (Kerns *et al*., 1983) and in male Wistar rats in a short-term exposure study (13 weeks) (Feron *et al*., 1988). Although the increased incidences of nasal tumours were not statistically significant in these last studies, they were considered exposure-related and biologically relevant because of the rarity of this type of tumour (NTP, 2011).

Studies on oral administration of FA in rats via drinking water showed to increase the incidence of fore-stomach tumours in male rats (IARC, 2006). Moreover, increased incidences of rare malignant gastrointestinal tumors were observed in female Sprague-Dawley rats exposed to FA *in utero* and throughout life via drinking water (Soffritti *et al*., 1989; IARC, 2006). In a similar study, the life-long ingestion of FA caused increased incidences of total malignant tumors in Sprague-Dawley rats including, testicular tumors in males, mammary-gland tumors in females and hemolymphoreticular tumors in both male and females of the high-exposure groups (Soffritti *et al*., 2002). IARC (2006) noted several limitations in these last two studies, namely the lack of historical control data, the misclassifications of lymphomas and leukaemia and discrepancies in tumour incidences.

No tumors were observed in skin by topical applications of FA solutions in mice (Iversen *et al*., 1986). With respect to other species, no tumors were found in monkeys exposed to FA by inhalation; probably because of the small number of animals exposed and short exposure duration (NTP, 2011).

2.5.4.3 ***Mechanisms of Carcinogenesis***

The mechanisms by which FA causes cancer are not completely understood, but most likely involve multiple modes of action, such as DNA reactivity, gene mutation, epigenetic effects, glutathione depletion, oxidative stress, chromosomal breakage, cytotoxicity and aneuploidy (NTP, 2011).

Upper airway cancers

Mechanistic studies in humans and experimental animals support the evidence that FA causes nasopharyngeal (IARC, 2006; NTP, 2011) and sinonasal cancer (NTP, 2011). The existing data strongly indicate that genotoxicity plays a key role in the carcinogenesis of FA in nasal tissues and also that cellular replication (in response to FA-induced cytotoxicity) promotes the carcinogenic response of the upper airway tissues (Conaway *et al*., 1996; McGregor *et al*., 2006).

Indeed, several studies show that inhaled FA is capable of causing genetic damage in the nasal epithelium of both experimental animals (e.g. DNA- protein cross-links above 6

ppm) and humans (e.g. buccal and nasal MN) (Conaway *et al*., 1996; Viegas *et al*., 2010; Costa *et al*., 2013).

Moreover, computational fluid dynamic model studies that have predict and compare FA-local airflow in the nasal passages of experimental animals showed that airway deposition and cytotoxicity-induced by cellular proliferation are important variables in the carcinogenicity of FA to nasal cells (Kimbel *et al*., 2001).

In rats, regional FA flux was correlated with the anatomical distribution of FA-induced lesions (squamous metaplasia) and DNA-protein crosslinks (Georgieva *et al*., 2003). FA-cytotoxicity in the nasal epithelium may result in cellular proliferation and in the promotion of chemically induced spontaneous mutations which may contribute to a carcinogenesis process (Monticello and Morgan, 1997). Indeed, cell-proliferation rates were correlated with the site specificity of nasal tumors (Monticello *et al*., 1989; Monticello and Morgan, 1997). However, FA exposure also causes cytotoxicity and cellular proliferation at anatomical sites that are not thought to be the origin of the squamous-cell carcinoma, suggesting that factors other than cellular proliferation may play a role in FA-induced nasal cancers (NTP, 2011).

Lymphohematopoietic cancer

As discussed above, several epidemiological studies found evidence of an association between exposure to FA and leukaemia. These findings have raised some discussion in the scientific community. Owing to its reactive nature it has been considered unlikely that inhaled FA is able to damage directly the stem cells in bone marrow as classical leukaemogens and cause leukaemia (Zhang *et al*., 2010a). In fact, some authors strongly stated that the fact of inhaled FA enter systemic circulation, reach bone marrow and cause leukaemia is biologically implausible and FA genotoxic and carcinogenic action are limited to local effects, in the area of first contact where is rapidly metabolised (Heck and Casanova, 2004; Franks, 2005; Speit, 2006). In addition, some studies have showed that the endogenous concentration of FA in the blood of humans, monkeys, and rats (2-3 μg/g) does not increase after inhalation (Heck and Casanova, 2004). Furthermore, the biological evidence of systemic (geno-) toxic effects of FA in both animal and human studies is still insufficient and conflicting, with both positive and negative outcomes (IARC, 2006, 2012).

However, it has been previously suggested that FA could potentially reach bone marrow directly in its hydrate methanediol form. Although endogenous FA is usually rapidly metabolised by GSH-dependent pathways, excess levels such as those encountered

occupationally could in fact saturate metabolic capacity and lead to genotoxicity in DNA and chromosomes (Zhang *et al*., 2010b).

Additionally, there is consistent evidence for systemic distribution of FA in humans showed by increased levels of FA-albumin adducts in serum in occupational exposed subjects (Pala *et al*., 2008; Bono *et al*., 2010) and in smokers (Wang *et al*., 2009a), as well as, numerous studies in humans and experimental animals that have demonstrated that inhaled FA can cause hematologic effects, genotoxicity in circulating lymphocytes (Costa *et al*., 2008, 2011), and cancer at distal sites (e.g. myeloid leukaemia).

Nevertheless, the mechanisms by which FA causes myeloid leukaemia in humans are not known. A recent work by Zhang *et al*. (2009, 2010b) suggested two possible pathways by which FA could act as a leukaemogen in humans. One, by damaging hematopoietic stem/progenitor cells circulating in peripheral blood and the other, by damaging primitive pluripotent stem cells present within the nasal turbinates and/or olfactory mucosa (Zhang *et al*., 2009). In both models, the damaged stem/progenitor cells would then travel to the bone marrow and become initiated leukemic stem cells, potentially developing into leukaemia following a latency period (Zhang *et al*., 2010b). Given the likely dynamics of stem cell turnover between the nasal/oral passages, blood and bone marrow, sufficient stem cells could be targeted through these two alternative models, focused around genotoxicity, to induce leukaemia, under conditions of chronic high FA exposure (such as occupational exposure) (Zhang *et al*., 2009).

Overall, the available evidence taken together does not indicate that such mechanisms are implausible (NTP, 2011; IARC, 2012). New *in vitro* data demonstrate FA's ability to induce genotoxicity in mouse erythropoietic cells and suppress human erythroid progenitor cells expansion supporting the potential of adverse effects of FA on hematopoietic stem/progenitor cells (Ji *et al.*, 2014). Furthermore, MN formation in mouse erythrocytes occurred in a dose-dependent manner, suggesting that chromosomal damage may be one potential mechanism underlying FA induced leukemogenesis. Additionally, it was recently reported that a FA-detoxifying enzyme (ALDH2) is involved on aldehyde detoxication in haematopoietic stem cells (Langevin *et al*., 2011; Garaycoechea *et al*., 2012). Thus, the biological plausibility of FA-induced leukemogenesis cannot be excluded.

2.7 EXPOSURE TO FORMALDEHYDE IN PORTUGUESE WORKPLACES

To our knowledge, Mayan *et al*. (1995) conducted the first study in Portugal on occupational exposure to FA. The aim was to assess the working conditions of health

professionals exposed to FA from histology, pathology and anatomy laboratories and to evaluate the neurobehavioral impact of such exposure on workers. Results on neurobehavioral indices were inconclusive. However, the values of FA concentration found in the labs were near or above 1 ppm, the higher levels were obtained during specific pathology and anatomy tasks. A later study by Ferro *et al*. (2005) confirmed these findings in five hospital pathology anatomy laboratories located in Lisbon. Similarly, an independent study conducted in the north and centre of Portugal found that the level of exposure of these professionals exceed the national recommended criteria of 0.3 ppm (Costa *et al*., 2008). Furthermore, in this study, the authors also evaluated the biologic effects of FA-exposure and for all the genotoxic endpoints studied significant increases were found in FA-exposed group compared with controls (Costa *et al*., 2008). These findings were further replicated in two other studies on FA-exposed health professionals and industrial workers (Viegas *et al*., 2010; Ladeira *et al*., 2011).

In a survey to assess the symptomatology of a group of medical students exposed to FA during an anatomy class (2h) most students reported symptoms of eye (96%), nose (83%) and throat irritation (65%) (Almeida *et al*., 2000) in accordance to described elsewhere (Takahashi *et al*., 2007; Wei *et al*., 2007).

Viegas and Prista (2007) in order to identify a possible relationship between occupational exposure to FA and nasopharyngeal cancer investigated by means of personal interviews the occupational history of a group of patients of the Portuguese Institute of Oncology diagnosed with nasopharyngeal cancer. In spite of some limitations, identified by the authors, the study allowed to verify a possible relationship between the exposure to this aldehyde and the development of the considered effect.

II. Present Study

1. **AIM**

Exposure to a chemical carcinogen involves a continuum of events from absorption, metabolism, DNA damage and DNA repair, resulting, in the worst case, in the development of cancer (Perera and Weinstein, 2000). Various biomarkers may be used to follow these events in order to elucidate the mechanisms of the genotoxic/carcinogenic process as well as the individual response to hazard chemical compounds.

The prime objective of the present study was to evaluate the occupational exposure to formaldehyde (FA). In particular, to assess the potential genotoxic effects of FA exposure, through differentiation of external *versus* internal levels and taking into account individual susceptibility (polymorphic genes in xenobiotic metabolising and DNA repair pathways) on the biomarkers of effect.

Therefore the proposed objectives for this study were:

i. To estimate the mean level of environmental FA exposure to which the anatomical pathology laboratory workers were exposed during their work shift;

ii. To investigate the use of formic acid in urine as a biomarker of exposure to FA;

iii. To assess if exposure to FA could cause cytogenetic alterations in peripheral blood lymphocytes (PBLs) evaluated by means of chromosomal aberrations (CAs), sister-chromatid exchange (SCE) tests, and DNA damage, evaluated by means of comet assay;

iv. To evaluate if FA exposure could cause cytogenetic alterations measured by means of micronuclei frequency in exfoliated buccal cells, a first contact tissue, and in PBLs, a systemic tissue;

v. To assess if FA exposure could induce somatic mutations evaluated by T-cell receptor mutation assay (TCR-Mf);

vi. To examine if FA exposure could influence the major lymphocyte subsets, namely total T lymphocytes (%$CD3^+$), T-helper lymphocytes (%$CD4^+$), T-cytotoxic lymphocytes (%$CD8^+$), B lymphocytes (%$CD19^+$) and natural killer (NK) cells (%$CD16^+56^+$);

vii. To clarify the potential role of the genetic polymorphisms in genes related to the metabolic pathway of FA (*CYP2E1*, *GSTM1, GSTT1, GSTP1*) and the repair of DNA lesions (*FANCA, RAD51, XRCC2, XRCC3, XRCC1, PARP1, MUTYH, BRIP1)* in modulating individual levels of biomarkers related to FA exposure.

In all the exposure and effect biomarkers studied, the role of potential confounding factors (age, diet, gender and smoking status) was also evaluated.

2. STUDY POPULATION

2.1 GENERAL CHARACTERISATION

Study population consisted of 172 subjects, 85 workers exposed to formaldehyde (FA) from nine Hospital Anatomical Pathology laboratories, located in the North and centre of Portugal working for at least one year, and 87 non-exposed control employees working in the same area in administrative offices and without occupational exposure history to FA.

Health conditions, general medical history, medication, diagnostic tests (X-rays, etc.) and relevant individual information such as, age, smoking habits and alcohol consumption, were assessed by means of questionnaires. The questionnaire (Annex I) also included information on dietary habits according to Bonassi *et al.* (2011). Subjects of the exposed group also gave information related to working practices namely use of personal protective equipment, years of employment, specific symptoms related to FA exposure and chronic respiratory diseases such as asthma or others. All individuals who agreed to participate in the study were fully informed about the procedures and objectives of the work in progress and each subject prior to the study signed an informed consent form. Ethical approval for this study was obtained from the Ethical Board of the National Institute of Health.

The general characteristics of both groups are described in Table II.

Table II. Characteristics of the study population

	Controls	**Exposed**	
	(N=87)	***(N=85)***	***p-value***
Gender			0.921[b]
Females	67 (77%)	66 (78%)	
Males	20 (23%)	19 (22%)	
Age (years) [a]	38.9 ± 11.0	39.8 ± 9.5	0.571[c]
Years of employment [a]		12.0 ± 8.2	
Smoking Habits			
Non-smokers	65 (75%)	64 (75%)	
Smokers	22 (25%)	21 (25%)	
Years smoking [a]	21.7 ± 11.3	20.4 ± 11.0	0.704[c]
Cigarettes/day [a]	13.7 ± 6.9	11.0 ± 5.8	0.160[c]
Packs/year [a]	14.8 ± 11.5	11.2 ± 8.1	0.248[c]

[a] Mean± standard deviation
[b] Chi-square test (bilateral)
[c] *t*- Student test

Smoking habits groups were established as non-smokers and smokers, since the number of ex-smokers (subjects that stop smoking for at least 2 years) was low and the average years as ex-smokers was higher than 9 years (controls,17 years; exposed, 10 years).

Data regarding dietary habits namely the daily consumption of tea, coffee, fruits, vegetables (salad and soup), protein intake (fish and meat) and vitamin supplements show similar dietary habits in control and exposed groups (Figure 4).

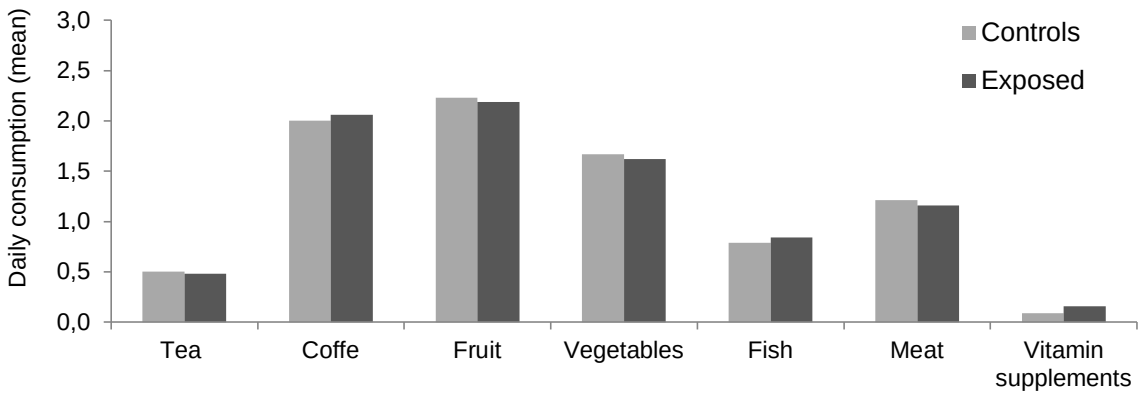


Figure 4. Frequency of the daily consumption of cups of tea/coffee, fruit (units), vegetables/fish/meat (meal) and vitamin supplements (intake) by study groups.

2.1.1 **Contact with study subjects**

The hospital units were identified through the National Healthcare Service web portal. Fifteen institutions with Anatomical Pathology Departments were selected within the desired geographic areas. A brochure with a brief description of the study and aims (Annex II) accompanied the first contact by letter. Besides promoting the study and generating interest, the main objective was to seek institutional approval to conduct the study and authorisation to contact the target professionals. Nine hospitals agreed to participate, two declined, one was not included in the study by time restraints, and three did not respond to contact. After institutional consent, the second step was the direct contact with the professionals to describe the project and to clarify any questions about the study and the nature of the participation required. A second visit was necessary to characterise the workplace and identify additional monitoring sites relevant for subsequent FA air evaluation. Overall, 63% of the workers agreed to participate in the study. The same procedure was followed for control subjects.

2.1.2 **Professional activity (brief description)**

Specialised and non-specialised health professionals compose a Hospital Anatomical Pathology Department. The non-specialised professionals include hospital orderlies (cleaning of equipment and facilities) and administrative staff (logistic activities, reception of biological specimens). The specialised health professionals comprise anatomo-pathology physicians and anatomo-pathology technicians. In general, there are four main lab areas or activities namely cytology, histology, macroscopic analysis (or registry) of biopsy specimens and microscopic examination. Anatomo-pathology physicians are mainly engaged in macroscopic and microscopic examination of biological specimens, whereas anatomo-pathology technicians are allocated to all lab activities mentioned above. Depending on the task and work location in the lab, all four professional categories are potentially exposed to FA.

The distribution by professional activity of the FA-exposed workers participating in this study is shown in Figure 5.

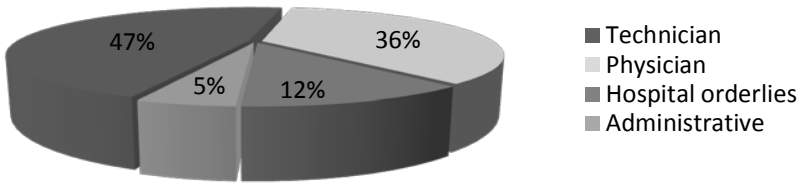


Figure 5. Distribution ratio (%) of the FA-exposed workers by professional category.

2.2 **BIOLOGICAL SAMPLES COLLECTION**

All biological samples were coded and analysed under blind conditions. All biological samples were collected simultaneously. Venous blood samples (15 mL) were collected from each donor, between 10 and 11 am. Prior to buccal cells collection subjects were asked to rinse the mouth with tap water to removed unwanted debris. For every subject, an individual sample from each cheek (left and right) was collected, and suspended in buccal cell buffer. Both cheeks were sampled using separate cytobrushes (Deltalab). A spot urine sample was also collected at the end of the shift in sterile polyethylene containers (50mL) and frozen at –20 ºC until analysis. After collection, all biological samples were transported in a cooler (4 ºC, transport within 2h maximum). Blood samples and buccal cells suspensions were immediately processed in the laboratory for the different methodologies used in the study.

2.3 STATISTICAL ANALYSIS

A general description of the study population was performed through univariate analysis. The distribution within the study groups of socio-demographic and lifestyle factors was evaluated with the Student's *t*-test for continuous variables and the Pearson's Chi-square test for categorical variables.

The effect of exposure was preliminarily assessed by Student's *t*-test. A logarithmic transformation of the data was applied to the following variables: urinary formic acid (FMU), percentage of DNA in the comet tail (% TDNA), TCR-Mf (T-cell receptor mutation frequency), $CD4^+/CD8^+$ ratio and B lymphocytes ($\%CD19^+$) to achieve a better approximation to the normal distribution. No transformation was needed for SCE (sister chromatid exchange), total T lymphocytes ($\%CD3^+$), T-helper lymphocytes ($\%CD4^+$), T-cytotoxic lymphocytes ($\%CD8^+$) and natural killer (NK) cells ($\%CD16^+56^+$). As no improvement was achieved with transformation, the Mann–Whitney *U*-test was applied to all parameters obtained from chromosomal aberrations (CAs) test (total-CAs; chromosome-type aberrations, CSAs; chromatid-type aberrations, CTAs; gaps; aneuploidies; aberrant cells and multiaberrant cells), micronuclei in PBLs (MNL), micronuclei in exfoliated buccal cells (MNB) and nuclear buds in exfoliated buccal cells (BNbud).

A multiple regression analysis was performed to estimate the effect of exposure and possible confounders. For each biomarker, the method best fitting the data was chosen after an investigation of the data distribution. Linear regression was applied on the log-transformed %TDNA and all lymphocyte subsets; Poisson regression on non-transformed data was fitted for SCE, MNB, BNbud and multiaberrants, and on log-transformed data for TCR-Mf; lastly, negative binomial regression on non-transformed data was carried out for MNL, total-CAs, CTAs, CSAs, gaps, aberrants and aneuploidies. All models included age, gender and smoking habits, and parameter-specific actual confounders. Mean ratio (MR) was used as the point estimate of effect accompanied by its 95% confidence interval (CI).

Moreover, independent variables such as, alcohol consumption, vaccination status and oral intake of contraceptives or hormonal replacement were evaluated, however due to extremely small number of subjects or lack of responsiveness/ dubious answers these variables were not included in the final regression models.

An ancillary regression analysis was carried out to assess the effect of FA exposure level, exposure duration and professional activity, only in the exposed population. Adjustment for age, gender, smoking habits and actual confounders was applied. Since the personal

protective equipment (PPE) used by professionals (masks) was not the appropriate one for FA-airborne exposure this factor was not included in the analysis.

A possible role as effect modifiers of genetic polymorphisms, as candidate biomarkers of susceptibility, on the alterations induced by the exposure was also tested. As the number of homozygous variant individuals was low (or inexistent) for most genes studied, these were merged with the group of heterozygous subjects, assuming a dominant model for their inheritance. Mann-Whitney *U*-test was employed to test for associations between biomarkers of exposure and effect and a particular genotype. Differences in genotype distributions were evaluated by the Pearson's Chi-square test.

Associations between variables were analysed by Spearman's rank correlation. The level of statistical significance was set at 0.05. All analyses were performed using the IBM SPSS Statistics V. 20 software and STATA/SE 12.0 for Windows software.

3. AIR MONITORING AND BIOMARKERS OF EXPOSURE

3.1 OVERVIEW

3.1.1 Air monitoring (guidelines and standards)

The basis for the prevention and control of health risks due to occupational exposure to chemicals are guidelines and standards. Their primary aim is to protect human health from adverse effects of indoor exposure to air pollution and to eliminate or minimise exposure to agents that are or are likely to be hazardous. The guidelines are based on a comprehensive review and evaluation of the accumulated scientific knowledge available at the time of their development and often include expert judgment. They have the character of recommendations and may serve as scientific basis to assist policy-makers and regulatory authorities in setting legally binding limits, the standards. Other factors beyond the toxicological and epidemiological data are taken into account in setting regulatory standards. These factors include environmental, social, cultural and economic conditions, as well as technical feasibility. Nevertheless, governments and regulatory authorities may adopt the recommended limits as legally enforceable standards.

Whether it is guideline or standard, they represent the highest concentration in the air of a chemical under which it is believed that nearly all workers may be repeatedly exposed, day after day, over a working lifetime, without adverse health effects. The *nearly all* refers to individual susceptibility (genetic predisposition), social-demographic factors (age, gender, ethnicity, lifestyle) or others (previous exposures) that may induce an adverse biological response to a particular chemical at a concentration equal or lower than the limit level (ACGIH, 2013).

Three categories of occupational exposure limits (OELs) are usually used to classify the exposure: i) time-weighted average (TWA), concentration that should never be exceeded during workday (8h) or workweek (40h); ii) short-term exposure limit (STEL), concentration that should never be exceeded in 15 min exposure at any time during a workday; iii) ceiling (C), concentration that should never be exceeded during any part of working exposure (direct value). For some substances, a TWA alone or with a STEL is relevant, for others only a C-level is applicable, it depends on the toxicological properties of the chemical and nature of the work.

OELs are express in parts per million (ppm) or mg/m^3 for compounds that normally exist as a gas or vapour at room temperature and pressure.

A “healthy” workplace environment also includes providing instructions, procedures, training and supervision to encourage people to work safely and responsibly. When engineering controls or safe systems are not feasible or effective to reduce the risk to acceptable levels the use of personal protective equipment (PPE) as a control measure may aid to prevent the exposure and thus minimise the risk. Nevertheless, PPE does not eliminate/control the hazard at source and therefore if the equipment fails or if it is not suitable for the chemical in question the workers will not be protected.

3.1.1.1 ***Formaldehyde occupational exposure and regulation***

Occupational exposure standards and guidelines for airborne-formaldehyde (FA) set by countries or major international organisations are listed in Table III. The exposure limits recommended by the European Scientific Committee were also included (SCOEL, 2008).

FA-air concentration in workplace is legally regulated all over the world with defined TWA, STEL and C-levels of exposure ranging all three from 0.3 to 2 ppm (IARC, 2006).

Table III. Occupational exposure limits for airborne formaldehyde (standards and guidelines.

Country/Organisations	OELs (category)	*References*
China	0.4 ppm (C)	*Tang et al. (2009)*
European Commission*		
SCOEL	0.2 ppm (TWA) 0.4 ppm (STEL)	*SCOEL (2008)*
France	0.5 ppm (TWA) 1 ppm (STEL)	*AFSSET (2008)*
Germany	0.3 ppm (TWA) 0.6 ppm (STEL) 1 ppm (C)	*IARC (2006)*
Norway	0.5 ppm (TWA)	*IARC (2006)*
Portugal	0.3 ppm (C)	*IPQ (2007)*
Spain	0.3 ppm (C)	*IARC (2006)*
United Kingdom	2 ppm (TWA) 2 ppm (STEL)	*HSE (2011)*
United States of America		
ACGIH	0.3 (C)	*ACGIH (2013)*
OSHA	0.75 ppm (TWA) 2 ppm (STEL)	*OSHA (2011)*

* values recommended by the Scientific Committee on Occupational Exposure Limit Values (SCOEL). C, ceiling; TWA, time-weighted average; STEL, short-term exposure limit.

Over the time the expansion of scientific knowledge, production of new studies and the updating of evaluation criteria have led to the global review of the FA's occupational exposure limits (OELS) resulting in a reduction of the limit levels (Binetti *et al*., 2006). Nevertheless, although the number of scientific data on FA toxicity and potential cancer risk has increased in the last decade, the OELs in most countries remain the same (in some the values are not revised since the early 1990s, e.g. US, France), which impairs the establishment of more rigorous protective measures of the worker´s health. Thus, the review by policy-makers of the exposure limit values in light of recent implication of FA carcinogenicity is essential to minimise the risk.

Portuguese Standard 1796:2007 establishes the OELs of chemical agents. The OEL for FA is 0.3 ppm (IPQ, 2007), meaning the maximum safe FA-airborne concentration that should never be exceeded during any length of time.

3.1.2 **Biomarker of exposure-internal dose**

A biomarker of internal dose indicates that exposure to a particular compound has taken place by measuring the compound or its metabolite(s) in body fluids. Although human exposure to a particular chemical may be estimated by using workplace air monitoring there is individual variability in absorption, distribution, metabolism and excretion. Therefore, it is preferable to measure the actual amount of the compound, or its metabolite(s) in a tissue or fluid from an individual in order to estimate the actual exposure rather than the expected exposure. However, biomarkers of internal dose do not reveal to what extent the metabolised agent has affected the target tissues or cells.

3.1.2.1 ***Formic acid in urine as a biomarker of FA internal dose***

For occupational biomonitoring, the investigation of potential dose biomarkers that might give information on the actual exposure of the body to a specific chemical is of paramount importance. Ideally, any biomarker of exposure should be specific for the exposure of interest, detectable in small quantities, correlated with prior exposure, measurable by non-invasive techniques and able to be accurately quantified (Manini *et al*., 2007). The aim is to use them as a routine monitoring method allowing a quick assessment of the worker's exposure and if needed a rapid implementation of safety measures. In addition, it also provides information about the efficacy of any preventive measure already adopted.

Nowadays, sensitive analytical techniques allow accurate identification and measurement of stable elements with relative small sample volumes in a relative short time. Urine is the preferred biological matrix since it is non-invasive and easily accessible allowing a more

systematic and regular monitoring. Although variation in liquid intake and liquid loss may result in large differences in concentrations of substances in urine, this variation can be corrected by creatinine concentration of the urine (Herber *et al.*, 2001).

Over the years, methods for the determination of FA in blood (Lou *et al.*, 2001), breath (Lin *et al.*, 1995; Moser *et al.*, 2005), and urine (Takeuchi *et al.*, 2007) have been published. However, FA is a very unstable chemical molecule, highly reactive and readily metabolised, so its concentration in body fluids or expired air is not expected to be a reliable biomarker of exposure, even for acute exposures (ATSDR, 1999).

A more promising indicator for FA exposure is the level of formic acid in urine. After absorption, FA is rapidly metabolised by oxidation to formate. Formate is excreted in urine, as formic acid, or oxidised to carbon dioxide and exhaled. Therefore, theoretically the exposure to FA may potentially generate a shift in the formic acid levels in the urine of exposed individuals.

In fact, a few studies have reported higher concentrations of formic acid concentrations in urine of FA-exposed subjects (Schmid *et al.*, 1994; Cheng *et al.*, 2008b; Mautempo *et al.*, 2010). However, the suitability of the formic acid excretion in the urine as a parameter for the biological monitoring of exposure to FA is a quite controversial issue (Schmid *et al.*, 1994). Formic acid is a metabolite of other compounds (e.g. methanol) and an endogenous product of several metabolic pathways. In addition, a diet rich in carbohydrates and proteins (glycine) appears to alter its concentration in urine (Gottschling *et al.*, 1984). Human baseline concentrations of formic acid in urine may range between 6.5 mg/g creatinine and 23 mg/g creatinine (Heinzow and Ellrott, 1992; Schmid *et al.*, 1994; HCN, 2005; IARC, 2006).

An analytical method using gas chromatography with flame ionization detection (GC/FID) was developed and applied by Mautempo *et al*. (2010) for quantify formic acid in human urines. The sample preparation involves a derivatization process (with a methanolic solution) and solid phase micro-extraction (SPME). The developed method was shown to be selective, accurate, precise and linear for formic acid determination in urine of FA-exposed workers from an industrial unit.

For GC analysis of organic acids a derivatization step is generally needed to yield compounds more volatile and less polar (Vas *et al.,* 2004). This can be achieved by an esterification reaction of the acid with an alcohol, which involves a nucleophilic substitution usually catalysed by a strong acid (H_2SO_4) or a Lewis acid (Boucharat *et al.,* 1998).

Currently, the analytical techniques applied for extraction and concentration of volatile compounds from biological matrices are based on headspace analysis by solid-phase

micro-extraction (SPME). The non-solvent SPME technique first developed by Pawliszyn and co-workers integrates sampling, extraction, concentration and sample introduction into a single procedure (Pawliszyn, 1999). A number of reports are present in the literature addressing the usefulness of SPME analyses of drugs and xenobiotic substances in body fluids, including formic acid in urine (Lee *et al.*, 1999).

3.2 MATERIAL AND METHODS

3.2.1 Determination of Formaldehyde airborne concentration

Air sampling was performed in the workers breathing zone for representative working periods (60 min each sample) during formaldehyde (FA) specific related tasks. Other workplace sites considered relevant to the assessment were also sampled. Air sampling and FA analysis were performed according to the NIOSH method no. 3500 (NIOSH, 1994), with minor adjustments. All chemicals used were of analytical grade. Briefly, air samples were collected in impingers bottles with 1% sodium bisulfite solution connected to a battery-powered air sampling pumps operating at a flow rate of 1L/min. Each sampling was done in duplicate. An aliquot of the sampled solution (4mL) was reacted with 1% chromotropic acid solution (Merck) in the presence of H_2SO_4 (95%-97%, Merck) to form a purple mono-cationic chromogen. Freshly prepared standard solutions and blank followed the same treatment. The absorbance of the coloured complex was analysed using a UV/VIS spectrophotometer (ATI UNICAM) at 580 nm (Fagnani *et al.*, 2003). Analysis of the samples allowed the calculation of the eight hour (8h) time weighted average (TWA) level of exposure to FA for each subject.

3.2.2 Determination of formic acid concentrations in urine samples

Urinary formic acid was quantified according to Mautempo *et al.* (2009) with few modifications. Urinalysis was performed by gas chromatography with flame ionization detection (GC-FID).

Based on previous studies the agent selected for formic acid derivatization was methanol (Mautempo *et al.*, 2009). This reaction, in the presence of boron trifluoride (BF_3), yields methyl formate, the target analyte.

The extraction and concentration of the analyte was achieved by solid-phase microextraction (SPME). Several commercial fibres for SPME are currently available to extract volatile compounds. According to supplier recommendations (Supelco, Bellefonte, PA, USA) the fibre used in this procedure was coated with divinylbenzene/ polydimethylsiloxane (DVB/PDMS), 65 µm.

Samples and Standards preparation

All chemicals used were of analytical grade. Acetonitrile was the compound chosen as internal standard (Lee *et al.*, 1999; Berode *et al.*, 2000; Lee and Berode, 2005).

Prior to the analysis, the urine samples were thawed at 4 ºC and centrifuged (453x*g*, 5 min).

All calibration standard solutions were prepared in urine by different dilutions of an intermediary solution of ammonium formate salt (Alpha-Aesar), also in urine. These solutions were all freshly prepared. The working range of standard concentrations (5–50 mg/L) was selected based on the human baseline content of formic acid in urine reported in literature (Heinzow and Ellrott, 1992; Schmid *et al.*, 1994; HCN, 2005; IARC, 2006). All samples (and duplicates), standard solutions (and blank) and fortified test samples followed the same analysis procedure.

Procedure

To a 20-mL vial containing 4660 µL of urine and a small PTFE-coated stirring bar, was added 300 µL of BF_3-methanol 10% (w/w, Fluka) and 40 µL of acetonitrile solution 10mg/L (Merck). The vial was then sealed with a polypropylene cap with PTFE/silicon septum and stirred vigorously in a vortex mixer for 10 sec. This mixture was heated for 10 min on an aluminium block heater at 80°C. After derivatization the vials were cooled at room temperature.

Headspace-Solid Phase Microextraction

The piercing needle of the SPME device was passed through the septum. The sample was stirred (260 rpm) at 50°C on an aluminium block heater for 5 min to reach headspace equilibrium. The fibre was then exposed to the headspace for 10 min (260 rpm, 50°C) to allow adsorption of the interest compounds (methyl formate, acetonitrile). Afterward, the fiber was carefully pulled into the needle sheath and the SPME device was removed from the vial. It was immediately inserted into the GC port and exposed in the injection port for 1 min for complete desorption.

GC conditions and sample analysis

GC analyses were carried out on a ThermoFinnigan Model Focus gas chromatograph equipped with a flame ionization detection (FID) system.

The injection port of the chromatograph was installed with a glass liner appropriated for split analysis. The analyses were performed under the following chromatographic conditions: Varian WCOT silica capillary column, CP-Wax 57-CB, 25 m × 0.25 mm i.d, DF = 0.2 µm; sample injection in a split mode with 1:7 rate and 11mL/min flow; helium as the carrier gas at a constant flow of 1.5 mL/min. The FID temperature was 220 ºC, and the

injector temperature was 220°C. The oven temperature was 32 ºC followed by an increase of 5 ºC/min until 200 ºC. The total GC analysis run time was 12 min.

SPME fibre was conditioned in GC injector port in the beginning of analyses, between samples and at the end of each analysis session (8-10 injections) for 10 min, at 250 °C. Prior analyses allowed the identification of the interest compounds in the chromatogram by comparing their retention times with those obtained from reference samples, and by comparison with data reported in the Mautempo *et al.* (2009) study. Retention time for methyl formate and acetonitrile (internal standard) was respectively 1.9 min and 4.9 min.

Area peak ratios of methyl formate to internal standard were calculated for each sample, formic acid concentration was determined by linear regression performing calibration curves in the concentration ranges between 5–50 mg/L. As quality control criteria, only calibration curves with correlation coefficients equal or superior to 0.995 were accepted.

For each subject the formic acid concentration was corrected with the creatinine value and expressed as mg/g creatinine. Creatinine was determined using CREAJ Gen2 kit (Roche Diagnostics) on COBAS INTEGRA 800, according to manufacturer instructions.

Validation and acceptance criteria

The method was validated following the analytical performance parameters established by Eurachem validation guide (1998).

The line of best fit was determined by linear regression performing calibration curves in the concentration ranges between 5–50 mg/L. The variance across the linear range was statistically evaluated using the two limit concentration standards, 5 and 50 mg/L; no significant differences were found, the working range was well fitted. The linearity of the working range was also assessed and proved to be linear with a correlation coefficient of 0.999. Relative percentage difference (RPD) of inter-day and intra-day duplicate samples were equal or below 10%. Precision studies under repeatability conditions were also done across the working range. For each standard concentration the calculated coefficient of variation (CV%) (n=10) was equal or below 15%. The percentage of recovery (%R) of the analyte from fortified samples ranged between the acceptable criteria interval of 80%-120%. The method Limit of Detection (LOD) and Limit of Quantitation (LOQ) were estimated to be respectively, 0.8 mg/L and 2.6 mg/L. During method validation, parameters and acceptance criteria were defined for calibration slopes, correlation coefficients (≥0.995) internal standard peak areas and repeatability (RPD≤10%, CV≤15%).

3.3 RESULTS

3.3.1 Formaldehyde air monitoring and exposure biomarker

Analysis of air samples collected during FA-exposure allowed the calculation of the 8h-TWA (time weighed average) level of exposure for each subject. Urinalysis of formic acid concentration in samples from exposed and control subjects were performed to investigate this assay as a potential exposure biomarker for FA occupational exposure. Results of the air level of exposure to FA and the level of formic acid in urine (FMU) by study group are reported in Table IV.

Table IV. Results of FA-external exposure and biomarker of exposure

	Controls		**Exposed**		
	N	**mean ± SE** *(range)*	***N***	**mean ± SE** *(range)*	***p*-value**
FA-level of exposure (air) ppm	—	————	*85*	0.38 ± 0.03 *(0.08-1.39)*	
FMU (formic acid in urine) mg/g creatinine	*70*	11.53 ± 0.95 *(0-44.01)*	*63*	17.46 ± 1.33 *(1.16-52.79)*	0.002

The mean TWA-level of worker´s exposure to FA was 0.38 ± 0.03 ppm (range 0.08-1.39 ppm). The peak emission of FA occurred mainly during two routine tasks, macroscopic examination of FA-preserved specimens (average FA concentration in air 1.4 ppm; range 0.3-3.2 ppm) and disposal of specimens and waste solutions (average FA concentration in air 1.3 ppm; range 0.3-2.8 ppm). The current Portuguese occupational exposure limit is 0.30 ppm (ceiling level), meaning the maximum safe FA concentration that should never be exceeded during any length of time in a worker's breathing zone. Germany and the American Conference of Governmental Industrial Hygienists (ACGIH) also set for FA occupational exposure a maximum limit concentration of 0.30 ppm (TWA and ceiling, respectively). On the other hand, the Scientific Committee on Occupational Exposure Limit Values (SCOEL) appointed by European Commission has advised a 0.2 ppm (TWA) occupational exposure limit for FA in the workplace. The results obtained show that professionals of the anatomical pathology laboratories studied are exposed to levels of FA higher than admissible air standards and guidelines, both national and international.

Regarding the levels of formic acid in urine the results obtained showed a significant increase of this compound in FA-exposed workers compared to controls.

3.3.1.1 *Effect of exposure, lifestyle and work-related factors*

To understand the possible effect of recognised host factors such as gender, age and smoking habits on the dose biomarker concentration in urine (FMU) a multivariate analysis was applied. The influence of dietary habits described on chapter 2 (present study) was also evaluated but none of the parameters was found to be a confounder and therefore were not included in the final multivariable model. The data obtained is summarised in Table V.

The significant effect of exposure on FMU was confirmed with exposed workers showing an increase of 43% on the levels of urinary formic acid compared to controls. In addition, a borderline significant (p=0.05) increase was found among subjects with more than 45 years-old. In fact, a positive significant association was found between this biomarker and age (r=0.206, p=0.018) confirming this trend. No effect of gender or smoking habits were found.

Table V. Effect of exposure, gender, age and smoking habits on the levels of FMU with estimates of mean ratios (MR)

	FMU		
	N	**MR**	**[95% CI]**
Exposure			
Controls	*70*	1.00	
Exposed	*63*	1.43**	[1.12-1.83]
Gender			
Females	*101*	1.00	
Males	*32*	0.73	[0.54-1.00]
Age			
< 35 years	*49*	1.00	
35-45 years	*48*	1.26	[0.95-1.68]
> 45 years	*36*	1.37	[1.00-1.86]
Smoking Habits			
Non-smokers	*95*	1.00	
Smokers	*38*	1.01	[0.97-1.06]

******p<0.01

Concerning the exposed group no significant influence was observed on FMU levels of work related factors namely FA-levels of exposure (air), professional activity and time of exposure (data not showed). Further, no significant association was found between dose biomarker levels and work-related parameters.

3.4 **DISCUSSION**

In anatomical pathology laboratories, FA is a well-known compound traditionally used as a fixative and tissue preservative for over 100 years. Numerous studies have consistently shown that the levels of airborne-FA in these settings are often higher than the recommended limit values (IARC, 2012). Indeed, indoor air analysis performed by Keil *et al.* (2001) in anatomy laboratories revealed average daily room concentrations of airborne FA ranging from 0.5 to 1.5 ppm. Because of its sensitising properties, irritating effects and cancer implication, FA accounts probably for the most important occupational chemical exposure hazard concerning this professional group. Inhaled FA primarily affects the upper airways; the severity and extent of physiological response depends on its concentration in the air.

In the present study, the mean level of FA exposure found (0.38 ± 0.03 ppm) exceeds the current national recommended limit value of 0.3 ppm (ceiling). Furthermore, 56% of the professionals had levels of exposure higher or equal to 0.30 ppm, half of which were exposed to FA-concentrations above 0.5 ppm. Therefore, the data obtained show that professionals were exposed to levels of FA superior to both national and international workplace limit values (Table III). The main FA vapour emissions occurred during macroscopic examination of FA-preserved specimens and during the disposal of specimens and waste solutions. In most cases during these tasks, the professionals were only using facemasks for biological hazard, not appropriate to protect from FA vapours. The main reason given by professionals for not using goggles and the appropriate respirators (when available) was the interference in the efficiency of the activities taken, namely communication difficulties, taking notes and handling the biologic material.

Our finding is in accordance with previous studies reporting for these professionals high levels of exposure to FA, often above permissible exposure limit values. Shaham *et al.* (2002) reported for 14 pathology departments a mean low level and high level of FA exposure of 0.4 ppm and 2.2 ppm, respectively. Personal air sampling performed during a gross anatomy course by Akbar-Khanzadeh and Pulido (2003) revealed an individual FA-exposure ranging from 0.6 to 1.3 ppm. Ohmichi *et al.* (2006) also evaluated personal exposure levels and indoor FA concentrations in a gross anatomy laboratory during 3 lecture sessions, the average personal FA-exposure levels for instructors and students ranged between 0.4 and 1.1 ppm, while the room averages of FA concentrations varied from 0.4 to 0.7 ppm. Two recent Portuguese studies in anatomical pathology and histopathology laboratories reported a time-weighed FA level of exposure ranging from 0.05 to 0.5 ppm, and a mean ceiling FA concentration of 1.1 ppm (Viegas *et al.*, 2010; Ladeira *et al.*, 2011).

Overall, our data, in agreement with other studies, shows anatomical pathology laboratories as one of the occupational settings where workers are regularly exposed to levels of FA near or higher than recommended limit values, which indicates a potential risk to worker's health.

Given the above, implementation of security and hygiene measures, such as periodic air sampling and biological monitoring, as well as good practice campaigns may be crucial to decrease the risk associated to FA occupational exposure in anatomical pathology laboratories.

In this regard, the level of formic acid in urine was evaluated as a potential tool for biological monitoring of occupational exposure to FA.

In fact, our results showed that the concentration of formic acid in urine increased significantly in FA-exposed workers compared to controls, although no significant correlation was found with the FA- level of exposure.

Studies that monitor FA occupational exposure based on urinary formic acid shifts have also reported similar findings. In most studies, workers exposed to FA showed higher concentrations of urinary formic acid compared to controls (Boeniger, 1987; Mautempo et al, 2010) however, no consistent correlation was found between the formic acid concentrations in urine and FA exposure levels (Gottschling et al., 1984; Schmid et al, 1994). Some authors suggested that other external or individual factors beyond FA-exposure might influence the excretion of formic acid. Therefore, its suitability as a biological index for the biological monitoring of exposure to FA is a controversial topic.

To determine possible influencing factors on the physiological excretion of formic acid a group of subjects not occupationally exposed to FA was investigated. Schmid et al (1994) found a considerable inter and intra-individual fluctuations on urinary formic acid levels among participants. One possible influencing factor highlighted by the authors, although not assessed, was food composition. The excessive intake of carbohydrates and proteins appears to increase formic acid excretion levels (Boeniger, 1987). However, Heinzow and Ellrott (1992) detected no influence of dietary habits on formic acid excretion among a group of healthy adults.

In our study, the influence of dietary parameters in the level of urinary formic acid was also evaluated, but no effect was found, confirming previous results (Gottschling *et al.*, 1984; Heinzow and Ellrott, 1992).

The metabolic activity is other plausible factor contributing to the fluctuations observed. FA is not the only chemical precursor to endogenous formic acid formation, other compounds include methanol, acetone and halomethanes (ATSDR, 1999, Shaham *et al.*,

2003). However, it should be added that FA is a ubiquitous compound and metabolite of some common chemicals (e.g. methanol; ATSDR, 1999), therefore the variations obtained in non-occupational exposed subjects may still be influenced by FA.

Further, polymorphic genes in major FA-metabolic enzymes namely FDH, ALDH2 and ADH1 may influence the kinetics of FA metabolism (Teng *et al.*, 2001). A recent occupational study in China (Cheng *et al.*, 2008b) found significant differences in urinary formic acid excretion among workers exposed to FA carrying different genotypes of *ALDH2* polymorphic gene. Moreover, data showed that FA exposure concentration and *ALDH2* genotypes may influence the urinary formic acid increment. This study clearly indicates an association between FA exposure levels in the workplace and formic acid excretion in urine, expressed by specific FA-enzymatic metabolism.

Concerning the influence of gender, age and smoking habits on urinary formic acid concentrations, a positive association was found only with age. This effect was also observed in a previous study (Heinzow and Ellrott, 1992) and may be related to micronutrient status (e.g. folate) or disturbance on the tetrahydrofolic acid pathway (Boeniger, 1987) with increasing age.

In summary, under the present state of knowledge, the level of formic acid in urine should be used to estimate occupational group exposures to FA, rather than individual exposures, since its measurement effectively indicated the exposure to FA, but was not correlated with the FA-level of exposure.

4. BIOMARKERS OF EFFECT

4.1 OVERVIEW

Biomarkers of effect are biological indicators of the body's response to exposure and indicate early sub-clinical changes, which if sustained, may go on to have pathological consequences (Links *et al.*, 1995). They either indicate early processes preceding disease or predict the development and presence of disease (by altered structure and/or function) (Kyrtopoulos, 2006). In terms of prevention, it is considered ideally if the biomarker is able to detect a biological alteration that is reversible. Therefore, although the early effect biomarkers are less associated with disease, their usage is much more useful in terms of disease prevention.

Biomarkers of effect include several markers, such as, reported gene mutations (e.g. HPRT, TCR gene mutation assays), altered gene expression (e.g., protein expression of metabolizing genes, DNA repair genes or specific enzymes), DNA strand breaks (quantified by comet assay) or cytogenetic alterations, namely chromosomal aberrations (CAs), micronucleus (MN) and sister chromatid exchange (SCE) (Au, 2007). They may also involve immunological responses (Lutrell *et al.*, 2008).

Cytogenetic endpoints have long been applied in surveillance of human genotoxic exposure and early effects of genotoxic carcinogens. Assays measuring CAs, MN and SCEs in lymphocytes are well-established techniques used extensively in human biomonitoring studies to assess DNA damage at the chromosomal level. The relevance of cytogenetic alterations as a cancer risk biomarker is further supported by epidemiologic data linking CAs and MN with cancer risk in human populations (Hagmar *et al.*, 1998; Bonassi *et al.*, 2007).

During the last years, the single cell gel electrophoresis assay or comet assay has been proven to be a very sensitive tool in human biomonitoring for the detection of different levels of DNA damage at the individual cell level (Collins, 2004). The simultaneous use of cytogenetic tests and comet assay in occupational studies allows a comparison of the presence of DNA strand breaks due to both acute and chronic exposure and of chromosome damage due to clastogenic and aneugenic events. In the case of chronic exposure, comparing the levels of damage from a cytogenetic technique with the comet assay could provide information about past versus current exposure (Albertini *et al.*, 2000).

Critical for using cellular phenotypes as biomarkers in human populations is the selection of biological specimens to be investigated (Mateuca *et al.*, 2006). The general approach is assess these endpoints in readily available surrogate cells to estimate events occurring in target tissues and to provide early warning signals for adverse health outcome. The most frequently used sentinel cells in human studies are peripheral blood lymphocytes (PBLs). The main reason for using lymphocytes is that these cells circulate throughout the body and have reasonably long life-span; therefore, they can be damaged in any tissue/organ-specific toxic environment (Au, 2007). Furthermore, assuming the mechanisms of chromosome damage formation are similar in the different tissues, the level of damage in PBLs can be expected to reflect the level of damage in cancer-prone tissues and to indicate cancer risk (Norppa *et al.*, 2006). Their preference is also associated with the relatively easy harvest, the large number in the human body and resistance even after exposure to cytotoxic agents (Ramalho *et al.*, 1995).

In recent years, other non-blood cells obtained by non-invasive methods, such as buccal, nasal or urothelial cells have received a large interest by the scientific community. These cells are easily and rapidly collected and in some cases are better models than lymphocytes, since they are target tissues of some reactive chemicals. There has been some debate about whether the biological events observed in surrogate cells also occur in target cells and whether the observed events predict cancer or not. For particular exposures, when possible, using biomarkers of effect that can be evaluated both in target tissue as in surrogate tissue would help to clarify these questions (Salama *et al.*, 1999).

One of main issues concerning biomarkers of effect is the interpretation of altered levels of these indicators at individual level. Traditionally, risk predictions are only valid at a group level and therefore effect of inter-individual variability is removed (Bonassi and Au, 2002). Also of note, the detection of a biomarker not necessarily indicate the presence of a disease, it may only indicate an effect of exposure of the organism to a substance.

4.1.1 **Chromosomal Aberrations**

Chromosomal aberrations (CAs) are changes in normal chromosome structure or number that can occur spontaneously or as a result of chemical or radiation exposure (Mateuca *et al*., 2006).

The frequency of structural CAs in peripheral blood lymphocytes (PBLs) has been used for decades as a biomarker of the early effects of genotoxic carcinogens in occupational and environmental settings (Norppa *et al*., 2006).

Structural CAs may be induced by direct DNA breakage, replication on a damaged DNA template, inhibition of DNA synthesis or other mechanisms (e.g. topoisomerase II inhibitors) (Albertini *et al*., 2000). Usually, structural CAs are divided into two broad categories, chromosome-type aberrations and chromatid-type aberrations visible in metaphase-arrested cells (Savage, 1975). Although each class differs from each other morphologically and mechanistically, both include breaks and exchanges being breaks the more abundant CAs found in peripheral lymphocytes (Norppa *et al.*, 2006), since they may also occur "spontaneously" at a low level (Savage, 2004)

While morphologically chromosome-type aberrations (CSAs) involve the same *locus* on both sister chromatids on one or multiple chromosomes, chromatid-type aberrations (CTAs) affect only one of the sister chromatids on one or more chromosomes (Suspiro and Prista, 2011).

Mechanistically, the formation of structural CAs requires one or several DNA double strand breaks (DSBs). The main differences between CSAs and CTAs formation is the timing and the type of initial lesion, and the DNA repair mechanisms (Hagmar *et al*., 2004).

Depending on the inducer agent, DSBs may result directly or indirectly by preexisting DNA lesions. Generally, the lesions are repaired by three main mechanisms: homologous recombination repair (HR), non-homologous end joining (NHEJ) repair and single-strand annealing (SSA). HR is an error-free repair process, highly accurate that precisely restores the original sequence at the break. NHEJ is an error-prone repair process that joins directly the two broken ends which usually leads to small-scale alterations at the break site. SSA mainly leads to the formation of interstitial deletions (Obe *et al*., 2002).

CSAs are mostly generated *in vivo* in G0/G1 lymphocytes by S-phase independent clastogens and reflect DSBs which are incompletely repaired or unrepaired by the NHEJ repair and SSA mechanisms. After DNA synthesis and chromosome duplication, the CAs formed in G0/G1 are doubled and chromosome-type breaks and exchanges (e.g. dicentric and ring chromosomes) are seen in metaphase (Figure 6).

CTAs arise predominantly *in vitro* during the S-phase of the cultured lymphocytes, in response to base modifications and single-strand breaks induced *in vivo* by S-phase-dependent clastogens. DSBs are formed from these initial lesions during DNA replication (Figure 7). Incomplete or failed repair of these lesions by conservative HR will trigger CTAs formation in a subsequent metaphase (Albertini *et al.*, 2000; Hagmar *et al.*, 2004).

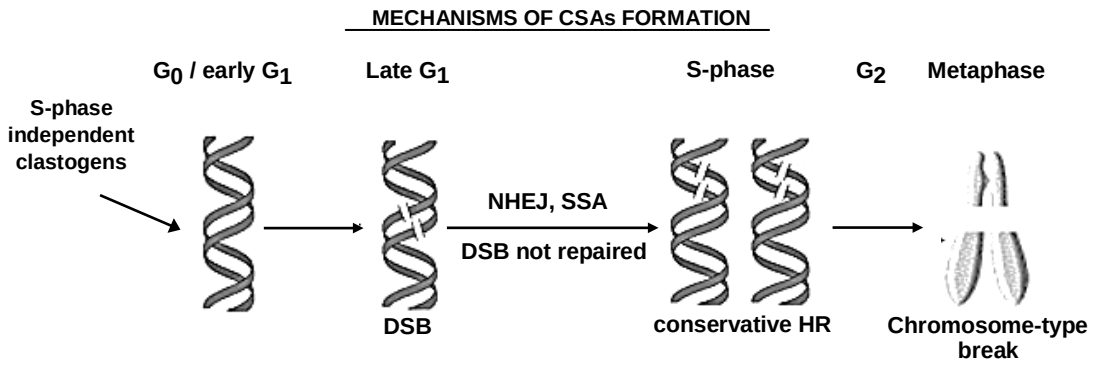


Figure 6. Example of formation of a Chromosome-type aberration (CSAs) (*adapted from Mateuca et al., 2012*)

The type of chromosomal aberration will be decisive for the fate of the cell. DSBs lesions are potential inducers of carcinogenesis through activation of proto-oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity (Obe *et al*., 2002). Cells with unstable aberrations that can pose problems on transcription, replication and chromosome segregation are eliminated by apoptosis. Stable aberrations (e.g. deletions, translocations) on the other hand may have deleterious consequences for the organism since they are much less effective in causing apoptotic cell death.

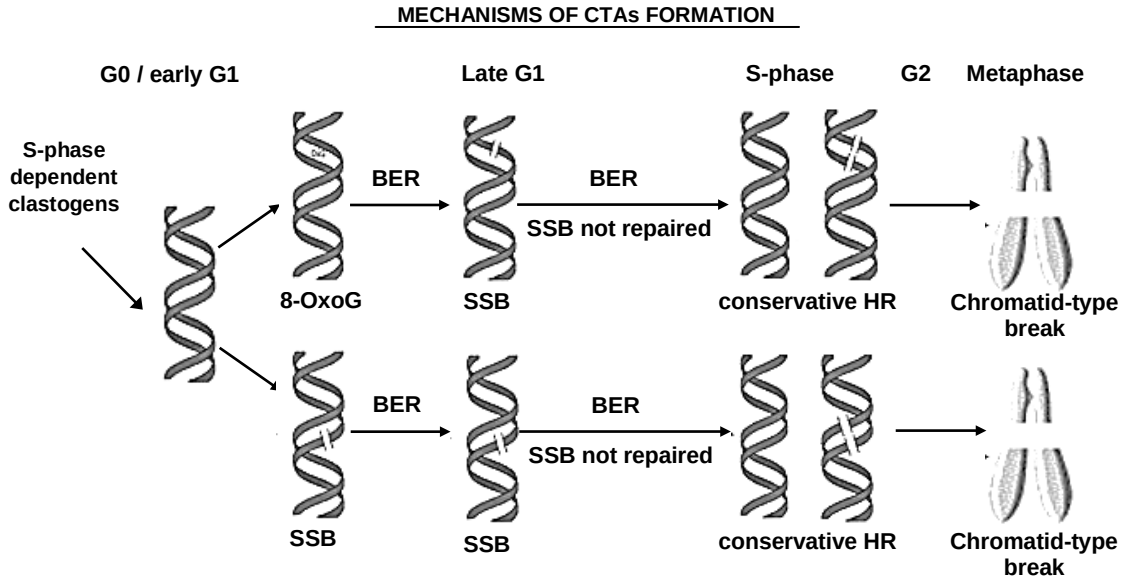


Figure 7. Example of formation of Chromatid-type aberration (CTAs) (*adapted from Mateuca et al., 2012)*

In human biomonitoring studies, detection of structural CAs is performed in PBLs by the CA assay. Information on the type of aberrations induced (S-phase independent versus S-phase dependent) following occupational and/or environmental exposure can be used to

identify into some extent the nature of the clastogenic damage produced via misrepair or misreplication (Palitti *et al*.,1998; Mateuca and Kirsch-Volders, 2007).

However, the CAs assay does not allow the detection of symmetrical chromosome-type rearrangements such as translocations and inversions. CSAs scored in humans are mostly chromosome-type breaks and to a lesser extent dicentric chromosomes or rings. CTAs are practically chromatid breaks, since chromatid-type exchanges are very rare (Norppa *et al*., 2006).

Among the biomarkers of early effect CAs is the most widely used and best validated biomarker (Bonassi and Au, 2002). This is because the mechanisms for CAs induction are better understood and because most environmental toxic substances have been shown to induce CAs (Au, 2007). CAs in PBLs are believed to represent a surrogate endpoint for more specific chromosome alterations in target tissues undergoing carcinogenesis.

Due to their extensive use and accumulated data it was possible to examine the potential association between structural CAs frequency and subsequent cancer outcome (Bonassi and Au, 2002). Indeed, CAs in lymphocytes revealed to be predictive of overall cancer risk in human populations (Hagmar *et al*., 1998). Both CSAs and CTAs were considered to have similar predictive values yet in some studies CSAs showed a stronger association (Norppa *et al*., 2006). During the last two decades, a number of epidemiologic studies showing that high CAs frequencies in PBLs of healthy individuals are associated with increased cancer risk (Hagmar *et al.*, 2004; Rossi et al., 2009) have enriched evidence concerning the role of CAs in carcinogenesis. The role of CAs on the etiology of cancer is further supported by the elevated frequencies of CAs found in most cancer cells and developmental abnormalities (Rossner *et al*., 2005; Au, 2007).

Numerical CAs refers to changes in chromosome number that occur due to abnormal cell division. Cells are classified as aneuploid when they contain an extra (hyperploid) or missing (hypoploid) chromosome. Chemically induced aneuploidy may arise by a variety of mechanisms which may include damage to the mitotic spindle and associated elements, damage to chromosomal sub-structures, alterations in cellular physiology and mechanical disruption (Bourner *et al.*, 1998; Albertini *et al.*, 2000). Aneuploid cells can be scored in metaphase preparations prepared for structural CAs analysis, although possible technical errors may occur during slide preparation. Therefore, assessment of aneuploidy is often considered a minor component of structural CAs studies, so only limited attention has been given to developing the most appropriate study design for this endpoint (Albertini *et al.*, 2000).

CAs assay has a key position in the test battery for genotoxic compounds and its protocol is defined by OECD guidelines (OECD 473). The combination with fluorescence in situ hybridization (FISH) chromosome painting methods can help to detect structural and numerical CAs and provide increased efficiency and specificity for identifying certain kinds of CAs induced *in vivo* (Albertini *et al.*, 2000).

4.1.2 **Micronucleus assay**

Micronuclei arise from acentric chromosome/chromatid fragments or whole chromosomes that are not included in the daughter nuclei at the completion of telophase during mitosis because they fail to attach properly with the spindle, lagging behind during the segregation process at anaphase. The displaced DNA material is eventually enclosed by a nuclear membrane and except for its smaller size is morphologically similar to the nuclei after conventional nuclear staining (Fenech *et al*., 2011). Consequently, micronucleus (MN) contains either chromosomal fragments or whole chromosomes.

Chromosomal fragments may result from direct double strand breakage, conversion of single strand breaks into DSBs after cell replication or inhibition of DNA synthesis (Albertini *et al*., 2000). In addition, misrepair of DSBs may lead to the production of a dicentric chromosome with an acentric fragment (Fenech *et al*., 2011). Frequently, at anaphase the acentric fragment results in a MN while the dicentric chromosome forms a nucleoplasmic bridge between the two daughter nuclei (Mateuca *et al*., 2006). Further evidence suggests that in these conditions some MN may also originate from the break of these anaphase bridges during cytokinesis (Fenech, 2006). Whole chromosomes are generally lag behind due to defects in the chromosome segregation machinery, such as deficiencies in the cell cycle checkpoint genes, failure of the mitotic spindle, kinetochore or other parts of the mitotic apparatus. Other causes may be associated to chromosomal substructures damage or hypomethylation of centromeric DNA (Albertini *et al.*, 2000; Fenech, 2006).

Once formed, a micronucleated cell may undergo apoptosis or may expel the MN, retain it in the cytoplasm or reincorporate it into the main nucleus. Nonetheless, the post-mitotic fate of MN and micronucleated cell is still a poorly understood event (Mateuca and Kirsch-Volders, 2007).

The use of MN as a biomarker of early genotoxic effects has become a standard assay in biomonitoring studies. In comparison with CA, the scoring of MN is simpler, requires shorter training and is less time consuming. MN assay is one of the best validated cytogenetic techniques for evaluating chromosomal damage in humans (Bolognesi and

Fenech, 2013). Since MN represents a measure of both chromosome breakage and chromosome loss, an increased frequency of micronucleated cells can reflect exposure to genotoxic agents with clastogenic or aneugenic modes of action (Albertini *et al.*, 2000).

Recently the *in vitro* mammalian cell MN test protocol for testing of chemicals was defined by OECD (OECD 487).

The further combination of MN assay with FISH probes allows the distinction between MN containing a whole chromosome (centromere positive MN) or an acentric chromosome fragment (centromere negative MN) contributing to a higher sensitivity and specificity of the method.

Besides its capacity to detect chromosome breakage/loss, the MN assay can provide additional parameters of genetic damage, namely nucleoplasmic bridges (NPB) and nuclear buds (Nbud). NPB generally results from dicentric chromosomes, when centromeres are pulled to the opposite poles of the cell at anaphase and are indicative of asymmetrical chromosome rearrangements and/or telomere end-fusions (Fenech, 2006). Nbud are characterised by having the same morphology as a MN, apart from being attached to the nucleus by a narrow or wide nucleoplasmic connection (Fenech, 2006). The process of nuclear budding occurs during S-phase and cells are sometimes referred as “broken egg” cells. Current evidence suggests that nuclear budding is the mechanism by which cells remove amplified and/or excess DNA and is therefore a marker of gene amplification and/or altered gene dosage (Fenech *et al.*, 2011).

MN frequency can be assessed in both peripheral blood lymphocytes (PBLs) or in exfoliated epithelial cells (buccal or nasal mucosa, or urine). MN can also be examined on erythrocytes but it is not a current practice (Albertini *et al.*, 2000). Frequencies of this biomarker can be influenced by age, sex, nutritional status or smoking habits (Fenech, 1998; Bonassi *et al.*, 2001). In human studies, PBLs are usually the most frequently used tissue for MN test. In occupational studies, this endpoint has been used successfully to assess the genetic damage in subjects exposed to potential carcinogens in the workplace.

4.1.2.1 ***Micronucleus assay in lymphocytes (CBMN)***

The cytokinesis-block micronucleus (CBMN) assay is the most extensively used method for measuring MN in cultured human lymphocytes because scoring is specifically restricted to cells in interphase that have divided once after mitogen stimulation. Since the induction of cytogenetic damage as MN can only be expressed in cells that have completed nuclear division, it is necessary to be able to distinguish these cells from resting cells (Mateuca *et al.*, 2006). By adding cytochalasin-B (actin-polymerisation

inhibitor) into the culture medium before the first mitosis the cytokinesis is blocked and once-divided cells are able to be identified by their binucleate appearance. Hence, CBMN assay allows to distinguish between mononucleated lymphocytes, which did not divide, and binucleated lymphocytes, which completed one nuclear division during *in vitro* culture and are able to expressed the inflicted damage *in vivo* (Farmer and Emeny, 2006). Consequently, the assessment of MN frequencies on binucleated cells represents measure of the damage accumulated before cultivation plus mutations expressed during the first *in vitro* mitosis (Mateuca and Kirsh-Volders, 2006). CBMN method also provides the opportunity to rapidly measure the progression of nuclear division (nuclear division index) by registering the number of mono, bi, tri and tetranucleated cells. Obtained result offers useful information regarding cytostatic potential of chemicals under study, identifying compounds that stimulate or delay cell division (Fenech, 1997).

Increased frequency of MN in lymphocytes of otherwise healthy subjects was recently associated to cancer risk (Bonassi *et al.*, 2007), indicating that MN formation is associated with early events in carcinogenesis. MN may originate from CTAs formed during DNA replication on a damaged template or CSAs initiated before the mitosis and duplicated at replication (Albertini *et al*., 2000). Hence, considering the cancer risk predictivity of CAs and the mechanistic similarities between CAs and MN formation, an association between MN frequency and cancer risk was somewhat expected (Mateuca and Kirsch-Volders, 2007). The link between MN induction in PBLs and cancer development is further confirmed by recent case control and meta-analysis studies (Iarmarcovai *et al*., 2008; Murgia *et al*., 2008). A significantly higher MN frequency was found in PBLs of subjects who developed cancer within 14 years after blood sampling (cases) as compared to those who were still cancer free at the end of the follow-up period (controls) (Murgia *et al.*, 2008). Moreover, the meta-analysis showed a 28–64% increase in the baseline MN level of untreated cancer patients compared to cancer-free referents (Iarmarcovai *et al.*, 2008). All the recently accumulated data on the cancer predictive value of elevated MN frequencies makes the CBMN assay a good candidate for wide application in human biomonitoring studies, especially those populations more susceptible to exposure to hazard chemicals. Furthermore, recent scientific evidence suggests an association between elevated MN frequency in lymphocytes and age-related degenerative and neurodegenerative diseases (Petrozzi *et al.*, 2002). Therefore, sensible biomarkers such as MN frequency that may identify individuals who are at an early stage of degenerative diseases would be useful since it would allow timely preventative intervention (Thomas *et al.*, 2007).

4.1.2.2 ***Micronucleus assay in exfoliated buccal cells (BMCyt)***

Other increasingly popular cell type used to assess the formation of MN in biomonitoring studies are exfoliated epithelial cells (urothelial, buccal or nasal cells). Exfoliated epithelial cells have several advantages regarding PBLs, are easily collected, minimally invasive and therefore do not cause undue stress to the study subjects. In some cases, these cells are better models than lymphocytes, since they are target tissues of particular cancers. Furthermore, approximately 90% of all cancers appear to have an epithelial origin (Rosin, 1992)

Buccal cells are the first barrier to numerous potential hazard substances entering the body through ingestion and inhalation, and are capable of metabolizing carcinogens to reactive products (Vondracek *et al.*, 2001; Spivack *et al.*, 2004). Therefore, exfoliated buccal mucosa (BM) cells are ideal to monitor early genotoxic events resulting from the specific exposure to these agents. MN assay in BM cells have been successfully used to show the genotoxic effects of lifestyle factors such as tobacco smoking and diet, medical treatments (radiotherapy) as well as occupational exposure to potential carcinogenic chemicals (Bloching *et al*., 2000; Burgaz *et al*., 2002; Holland *et al*., 2008).

The mechanism of MN formation in BM cells is the same of lymphocytes but the buccal micronucleus cytome assay (BMCyt) has a few advantages. The MN assay in a rapidly dividing tissue, such as the buccal mucosa, allows the assessment of DNA damage without the need for an *ex vivo* cell replication step, so the establishment of cell cultures required for CBMN or for classical metaphase analyses, such as CA assay and SCE test are not needed (Bonassi *et al*., 2011). The collection of buccal exfoliated cells from the inner wall of the cheek is minimally invasive and therefore this assay is well suited for large biomonitoring studies.

The buccal mucosa is composed of four layers of structural, progenitor and maturing cell populations, namely the connective tissue (*lamina propria*), the basal cell layer of active dividing cells (*stratum basale*), the prickle cell layer containing differentiated, apoptotic and necrotic cells (*stratum spinosum*), and the keratinised layer at the surface (*stratum corneum*). Finger-like structures called ‘‘rete pegs’’ project up from the *lamina propria* into the epidermal layer produces an undulating basal cell layer effect (Holand *et al*., 2008; Thomas *et al*., 2009). A photomicrograph of a cross section of normal buccal mucosa along with a diagrammatic representation of the different cell layers are presented in Figure 8.

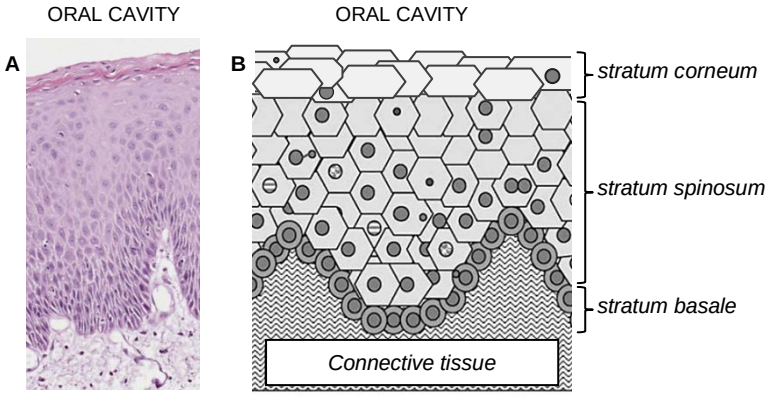


Figure 8. Photomicrograph (A) and a scheme (B) representation of a section of buccal mucosa showing the various cell layers (*adapted from Holland et al. (2008) and Thomas et al. (2009)*).

The oral epithelium maintains itself by continuous cell renewal by migration of new cells from the basal layer to the surface. The basal layer contains the stem cells that may express genetic damage as MN (chromosome breakage or loss) during nuclear division. These cells may also exhibit Nbud. However, the MN in buccal exfoliated cells is a relatively rare event, with a mean background frequency of 1 micronucleated cells every 1000 exfoliated cells (Ceppi *et al*., 2011). The daughter cells, which may or may not contain MN, in time differentiate into the prickle cell layer then into the keratinised superficial layer and finally exfoliate into the buccal cavity. Eventually, a few cells may be blocked in a binucleated stage and show two nucleus. Some cells may degenerate into cells with condensed chromatin, fragmented nuclei (karyorrhectic cells), pycnotic nuclei or completely lose their nuclear material (karyolitic cells). The molecular mechanisms leading to the various cell deaths events and their inter-relationships are unknown (Holland *et al*., 2008). Figure 9 illustrates the sequential spatio-temporal origins of the various buccal cell stages and the different genetic damage that can occur induced by exogenous agents such as inhaled carcinogens.

The evaluation of biomarkers of DNA damage (MN and Nbud) and cell death (condensed chromatin, karyorrhexis, pycnotic and karyolitic cells) provide a more comprehensive assessment of the cytogenetic damage induced by genotoxic agents. Therefore, the BMCyt assay may provide information on chromosome instability, cell proliferation (basal cell frequency), cytokinetic defects (binucleated cells) and cell death.

As before mentioned, it was recently established a positive association between MN in human lymphocytes and cancer risk. Regarding MN in BM the extent of epidemiological data available is quite limited because of its relatively recent adoption. Whether an elevated MN frequency in BM is predictive of cancer risk, or whether the relationship is primarily site-specific (oral cavity), or extends to overall cancer risk, remains for the

moment unknown. Validation and prospective studies on the BMCyt assay (Holland *et al*., 2008), as well as data on a strong correlation between MN in PBLs and BM, would help to evaluate this endpoint cancer predictability (Bonassi *et al*., 2011). Furthermore, similarly to MN in lymphocytes, recent scientific evidence suggests an association between BMCyt parameters and degenerative disorders, namely Alzheimer's disease (Thomas *et al*., 2007).

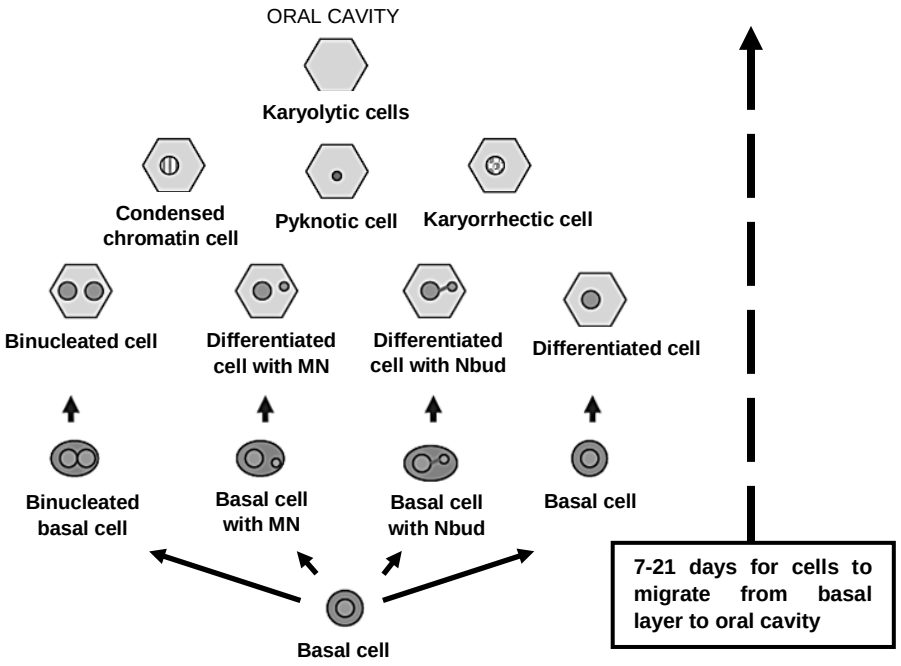


Figure 9. Diagrammatic representation of the sequential spatio-temporal origins of the different buccal cell-stages (*adapted from Thomas et al., 2009*).

4.1.3 **Sister-chromatid exchanges**

Sister-chromatid exchanges (SCEs) result from the reciprocal exchanges of DNA regions between two sister chromatids of a duplicated chromosome in response to a damaged DNA template during the S-phase of the cell cycle. The interchange process involves DNA breakage and reunion at apparently identical *loci* of the sister chromatids (Latt and Schreck, 1980). This process is considered to be conservative and error-free, since no information is generally altered during reciprocal interchange by homologous recombination (HR) (Wilson and Thompson, 2007). Although, its biological meaning and mechanism has not been fully elucidated, SCEs appear to be the consequence of DNA replication errors on a damaged template, possibly at the replication fork (Albertini *et al*., 2000). The mechanism by which SCEs are likely to occur involves the initial collapse of a replication fork when it encounters a pre-existing single-strand break (SSB) or gap in one parental strand (Wilson and Thompson, 2007). As a result, a replication-associated

double-strand break is formed with a 3' free end, which initiates the repair process via HR pathway and the invasion of the intact sister strand to serve as template for DNA synthesis. Resolution of the resulting Holliday junction by non-crossing over results in SCEs formation (Helleday, 2003) (Figure 10). Another possible pathway of SCEs induction involves the initial stop of the replication fork due to obstacles on the DNA template (e.g. adducts). The repair process of the stalled replication fork gives rise to a double-strand break with one free end, which can be further repaired by HR and may result, as described above, in SCEs formation (Helleday, 2003).

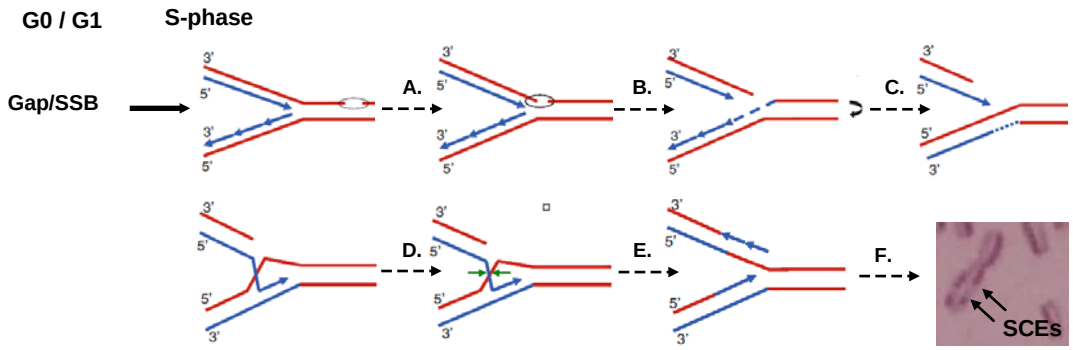


Figure 10. Mechanism of SCEs formation. A) Replication fork approaches a SSB. B) Collapsed replication fork; fork breaks; repair synthesis. C) 5' to 3' double-strand break resection. D) HR pathway, strand invasion. E) Resolution of the Holliday junction in the orientation shown by the green arrows results in SCE, as illustrated by the red/blue color junctions in the new "parental" strands. F) The replication fork is restored (*adapted from Wilson and Thompson (2007) and Mateuca et al. (2012)*).

Currently, the standard fluorescence assay used to visualise SCEs is based on the differential staining of the sister chromatids after two rounds of replication in the presence of 5-bromo-2´-deoxyuridine (BrdU) (Natarajan *et al*., 1994). BrdU is an analogue of the DNA thymidine that is efficiently incorporated into the DNA strands during replication. Because replication of DNA is a semiconservative process, when cells are cultured through a single replication cycle in the presence of this thymidine analogue, one DNA strand in each daughter chromatid is substituted with BrdU (Wilson and Thompson, 2007). After a second round of replication in BrdU medium, one chromatid contains one substituted DNA strand, while both strands of its sister chromatid are substituted. The chromatids can be further differentiated by treatment with the Hoechst dye, which fluoresces at a lower intensity when bound to DNA substituted with BrdU than when bound to unsubstituted DNA (Perry and Wolff, 1974). Following Giemsa staining the asymmetric distribution of BrdU-substituted DNA in sister chromatids can be observed by light microscopy. BrdU has the effect of "bleaching" so that the chromatid with more BrdU is lighter in appearance (Wilson and Thompson, 2007). Any SCEs appears as a

discontinuity of the stain along the chromatid. The differential staining of the chromatids is related to the photosensitisation by UV light used in the methodology which seems to preferentially break the bonds present in non-histone proteins of the chromatid containing more BrdU, giving rise to a lighter staining intensity of this chromatid (Mateuca *et al*., 2012). The SCE assay yields quantifiable data from every cell scored, which increases the efficiency of data collection and the identification of DNA damage resulting from exposure to genotoxic carcinogens (Albertini *et al*., 2000). In addition, scoring SCEs is more rapid compared to traditional chromosomal aberration analysis.

Upon the advent of non-radiolabel detection methods for SCEs, these cytogenetic alterations were applied as genetic indicators for potential genotoxins/mutagens in laboratory toxicology tests, since most forms of DNA damage induce chromatid exchange upon replication fork collapse (Wilson and Thompson, 2007; Rodriguez-Reyes and Morales-Ramirez, 2003). Indeed, SCEs showed to be efficiently induced by numerous mutagenic or carcinogenic agents, especially those which form covalent adducts to the DNA or interfere directly or indirectly with DNA replication (Natarajan *et al*., 1994).

This test has gained further popularity when it was noted that cells derived from patients with Bloom's syndrome, characterised by a high propensity for cancer, exhibit a hallmark high occurrence of SCE (Chaganti *et al*., 1974) likely due to dysregulation of HR by a defective helicase function (Wilson and Thompson, 2007). It is believed that defects in HR process may presumably be responsible for the genetic instability, increased SCE and high incidence of cancer in early life associated with this disorder. In fact, markedly increased SCE is used clinically as a diagnostic marker for Bloom's syndrome (Wilson and Thompson, 2007).

The readily quantifiable nature of SCEs with high sensitivity for detecting toxicant-DNA interaction and the demonstrated ability of genotoxic chemicals to induce a significant increase in SCEs in cultured cells have resulted in the application of this endpoint in human studies as a biomarker of chromosomal damage in PBLs of individuals exposed to genotoxic agents (Albertini *et al*., 2000).

This cytogenetic endpoint has revealed to be a highly sensitive tool for monitoring exposure to environmental contaminants in the workplace. Exposure to different chemical pollutants, including FA (Shaham *et al*., 2002; Costa *et al*., 2008), has been associated with increased frequency of SCEs (Teixeira *et al*., 2004 (styrene); Costa *et al*., 2006 (pesticides); Wang *et al*., 2012 (polycyclic aromatic hydrocarbons)).

Despite its good performance as a biomarker of effect to genotoxic/mutagenic chemicals, SCEs frequency does not appear to have cancer predictive value (Bonassi and Au 2002;

Norppa *et al*., 2006). A possible association between SCEs level and cancer risk is more difficult to evaluate compared to chromosomal aberrations since the baseline levels of SCEs showed to differ among individuals and across studies, which may partly due to technical variation (Norppa *et al*., 2006). Therefore, a general classification of subjects into a high, medium, or low SCE group is difficult and needs further investigation.

4.1.4 **Single cell gel electrophoresis (Comet assay)**

Comet assay or single cell gel electrophoresis (SCGE) is a versatile and simple method for measuring DNA damage and repair at individual cell level. It is based on the ability of negative charged loops/fragments of DNA to be pulled through an agarose gel in response to an electric field, appearing like a 'comet'.

During the last two decades, the comet assay became very popular and today is probably one of the most used tests for the assessment of DNA damage and repair. Although initially developed within a population of mammalian cells, comet assay applications now range from human and ecological biomonitoring (e.g., DNA damage in mussels living in polluted estuarine sites, earthworms in toxic waste sites or rodents near coal mines) to measurement of DNA damage in specific genomic sequences (Cotelle and Férard, 1999; Collins, 2004; Cortés-Gutiérrez *et al*., 2011).

Comet assay combines the simplicity of biochemical techniques for detecting DNA strand breaks, alkali-labile sites, and cross-linking, with the single cell approach typical of cytogenetic assays. The assay has also a few limitations, for example it is not able to detect aneugenic effects and epigenetic mechanisms (indirect) of DNA (effects on cell-cycle checkpoints) (Dhawan *et al*., 2009).

The main advantages of the comet assay include: (a) sensitivity for detecting low levels of damage, (b) single cell data collection, allowing more robust statistical analyses, (c) requirement for a small number of cells per sample, (d) low cost, rapid and ease of application (e) flexibility to use fresh or frozen samples and (f) use of any eukaryote monodispersed cell population, proliferating as well as nonproliferating, e.g. cells from tissue biopsies that can be homogenised, buccal cells, and also yeast, plant and sperm cells (with few modifications) (Piperakis, 2009; Azevedo *et al*., 2011). For most purposes, however, well-characterised cell lines or primary cells (e.g. peripheral blood cells) used in classical genetic toxicology testing assays, are preferred.

Several versions of the comet assay are currently in use being the most popular the alkaline version, first introduced by Singh and co-workers (1988). Briefly, cells embedded in agarose are placed on a microscope slide and lysed with detergent at high salt

concentration. During lysis, cellular membranes, cytoplasmic and nucleosplasmic constituents and most histones are removed. After lysis, what remains is the nucleoid containing RNA, proteins and negatively supercoiled DNA attached to the nuclear matrix. The immobilised nucleoid is then denaturated in an alkali buffer and electrophoresed in same buffer. Electrophoresis results in structures resembling comets, with a distinct head, comprising intact DNA and a tail of extended DNA loops and DNA fragments (Figure 11).

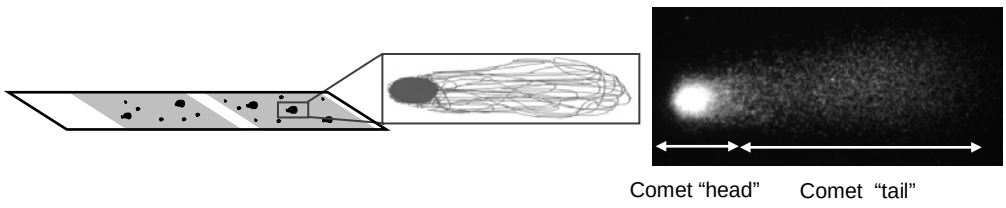


Figure 11. Schematic picture representing a comet of a damaged cell and image.

The "comet" can be visualised with a fluorescence microscope after staining with a DNA staining dye (e.g: ethydium bromide, acridine orange). The size and shape of the comet and the distribution of DNA within the comet correlate with the extent of DNA damage present in the individual cell (Figure 12).

Comets can be analysed by visual scoring, by computerised image analysis or by recently developed fully automatic computerised image analysis. There is a range of different software available to analyse comets. In visual scoring comets are divided into five categories (from class 0 to 4) representing increasing relative tail intensities and therefore increasing damaged cells (Collins, 2004). Computerised scoring is reported using a range of different parameters. The most frequently used endpoints are percentage DNA in tail (%TDNA), tail moment and tail length. The different ways of scoring cells have different advantages and limitations. Azqueta *et al.* (2011a) recently compared visual scoring, computerised scoring and automatic computerised scoring and concluded that all three scoring methods are reliable and results from the three methods are, to a certain extent, comparable.

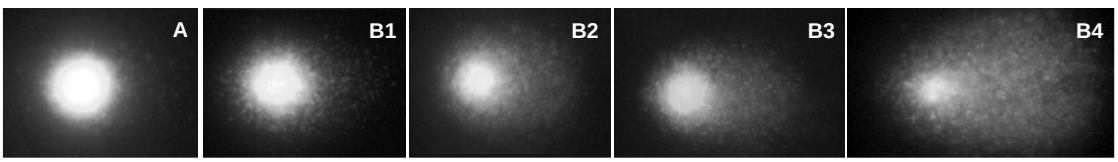


Figure 12. Comet assay images. (A) Untreated cell exhibiting intact DNA, without a tail. (B) Cells exhibiting increased DNA migration after treatment with a DNA damaging agent; images 1 to 4 represent different classes of DNA damage.

Some technical sources of variability were detected in the assay that can have an impact on the results such as the agarose gel density, electrophoresis time or voltage across the gel. Several guidelines have been published (Azqueta *et al.*, 2011a, 2011b), however a standardised protocol is still lacking.

During the last decade, the comet assay was introduced as a useful biomarker for risk assessment in human biomonitoring studies as an indicator of carcinogen exposure. It has been shown to be a valuable tool for acquiring knowledge about current levels of exposure to toxicants agents and for identifying hot spots or trends in exposure risks of human populations. Applications include monitoring occupational exposure to genotoxic chemicals or radiation, environmental exposure (air pollutants) and detection of DNA damage associated with smoking. Recently the comet assay has been used to investigate DNA damage and repair efficiency in a wide range of tumour cells in response to a variety of DNA-damaging agents. These studies include both investigations on human tumour cell lines and on tumour cells extracted from cancer patients. In addition, the Comet assay has also the potential to be used to aid in clinical diagnosis (e.g. xeroderma pigmentosum and Nijmegen breakage syndrome).

Besides is applicability to virtually any target tissue/cells, another reason for the increased use of the comet assay as a biomarker for risk assessment is the similarity of results between this assay and validated biomarkers extensively used in human studies (Kassie *et al*., 2000). Nevertheless, the usefulness of this assay depends on the toxicants involved, their molecular mechanisms of action, as well as the experimental design (e.g., use of control groups, timing of sample collection).

The growing popularity of comet assay among researchers along with the increasing awareness of its versatility and potential use raised the need to validate this assay as a valuable tool in risk assessment. In this sense, several collaboration efforts were made and a number of international workgroups and networks (ICCVAM, ECVAG, JaCVAM, ComNet Project) were established to address this important issue, within the current OECD regulatory strategy for genotoxicity testing. Recent international inter-laboratory studies have contributed greatly to the validation of this assay (Forchhammer *et al.*, 2010, 2012; Ersson *et al.*, 2013). The main objectives are to thoroughly evaluate the reliability and accuracy of the assay and to produce a standardised protocol with maximum acceptability by international regulatory agencies.

4.1.5 **T Cell Receptor mutation assay**

The T Cell Receptor (TCR) mutation assay detects somatic mutations, unlike other biomarkers of effect here described that provide information on chromosomal alterations. These types of mutations reflect genomic instability induced by inherited or acquired changes (Balmain *et al*., 2003) that result in easily recognised cellular phenotypic alterations (Albertini *et al*., 1993). Somatic mutations are also considered to constitute a biomarker of carcinogenesis, since a number of mutational events are thought to be necessary to convert a normal cell into a malignant cancer cell (Compton *et al*., 1991).

The TCR is a disulfide-linked heterodimer composed of two highly variable chains, α and β, that is expressed on the cell surface of the vast majority of peripheral blood T lymphocytes (Janeway *et al*., 2001). The genes coding for the two chains are located on chromosomes 14 and 7 respectively, and are phenotypically hemizygous. It is believed that only one of the two alleles is expressed actively as a result of allelic exclusion mechanism similar to that observed in the genes of immunoglobulins in B cells (Akiyama *et al*., 1995). Therefore, it is expected that a single mutation in the functional TCR genes results in the absence of phenotypic expression of the TCR on the cell surface.

The α:β TCR can only be expressed on the cell surface in complex with CD3 (cluster of differentiation 3). TCR and CD3 play an important role in antigen (small surface peptides) recognition and signal transduction so that the junction of the two components is critical to these functions (Clevers *et al*., 1988). If the expression of any of the TCR genes (α or β) is inactivated, the α:β TCR/ CD3 complex cannot be transported to the surface of the cell membrane and defective complexes accumulate in the cytoplasm (Akiyama *et al*., 1995). In addition, most of the T cell mutants obtained *in vitro* lacking surface expression of TCR/ CD3 were found to have defects in TCR expression rather CD3 suggesting that TCR genes are more susceptible to mutagenesis than CD3 genes (Kyoizumi *et al*., 1990).

TCR is essential to immune response. Through α:β TCR/CD3 complex T-cells specifically recognise protein antigens bound to the major histocompatibility complex (MHC) molecules at the surface of antigen-presenting cells. This ability to inspect degradation products of antigens creates a detection system that is intrinsically difficult for pathogens to avoid, as all proteins must eventually be degraded. It is also an extremely sensitive system, as very few peptide-MHC complexes on an antigen-presenting cell are needed to trigger a T cell response (Costa, 2012). The inactivation of a gene encoding a protein of the α:β TCR/CD3 complex should lead to loss of surface α:β TCR/CD3 expression and thereby result in defective antigen recognition and cell activation.

T cells, aside the α:β TCR/CD3 complex also express at the surface either CD4 or CD8 antigens. The $CD3^+CD4^+$ α:β T cells constitute 60% of the α:β T cells and, in general , are T-helper cells recognizing peptides bound to MHC class II molecules. $CD3^+CD8^+$ α:β T cells represent 30%; they are able to identify peptides (generated by endogenous processing) bound to MHC class I molecules and are mostly cytotoxic T cells (Costa, 2012).

A minority of T cells bear an alternative, but structurally similar, receptor composed by different polypeptide chains, γ and δ chain. γ:δ T-cell receptors appear to have different antigen-recognition properties than the α:β T-cell receptors, including nonpeptide ligands. The biological function of γ:δ T cells in immune responses is not yet entirely clear (Janeway *et al*., 2001).

The TCR mutation assay is a simple and quick method for evaluating the mutagenic effects in human populations requiring only a small volume of peripheral blood. The half-life of the majority of mutated cells is around 2-3 years (Taooka *et al*., 2006). Mutations in the TCR genes can be detected by flow cytometry using antibodies against CD3 molecules. The principle of the assay is to label lymphocytes with fluorescently tagged anti-CD3 and anti-CD4 antibodies to detect α:β TCR mutants in the population of T-helper cells expressing CD4; it measures the frequency of mutant cells $CD4^+CD3^-$ among normal $CD4^+$ T cells (Akiyama *et al*., 1995). This method considers the total number of mutations in the TCR chain genes, being unable to differentiate between mutations occurring in the α chain gene and occurred in the β chain gene.

The TCR mutation assay has been employed in human biomonotoring studies to detect the genotoxic effects of environmental mutagens, namely radiation exposure (atomic bomb and accidental events), tobacco use, chemotherapeutic exposure and mining contamination (Kubota *et al*., 2000; Taooka *et al*., 2006; Coelho *et al*., 2011). Occupational exposure studies includes exposure to lead (García-Lestón *et al*., 2011), vincristine (Jiang *et al*., 2008) and pesticides (Costa, 2012), but none, to our knowledge, assessed formaldehyde (FA) exposure.

4.1.6 **Immune markers: Lymphocytes subpopulations**

Evaluation of potential adverse effects on the immune system is an important component of the overall evaluation of a compound toxicity. The immune response is a complex process involving the interaction of various components from anatomical barriers to specialised cells. The interaction among the various cellular components of the immune system (i.e., lymphocytes, macrophages, granulocytes, and natural killer cells) is

extremely advantageous for the organism and through the continuing dialogue between innate and acquired immune response, efficiency is ensured. The immune system cells continuously undergo proliferation and differentiation; its main responsibility is the organism defence against invasion by pathogenic microbial agents and spontaneously arising neoplasms. In doing so, the intensity and specificity of the immune response must be highly regulated and capable of discerning self from no self (Luster *et al*., 1989).

A number of xenobiotic agents have the ability to alter the normal functionality of the immune system, potentially compromising the organism's ability to recognise, control or eliminate infectious agents or neoplastic cells (Veraldi *et al*., 2006). The sensitivity of the immune system to chemicals relies both on the general properties of the xenobiotic (e.g. reactivity with macromolecules) and on the complex nature of the immune system, which encompasses antigen recognition and processing; cellular interactions involving cooperation, regulation, and amplification; cell activation, proliferation, and differentiation; and mediator production (Luster *et al*., 1989).

Due to the structural and functional complexity of the immune system, the identification of substances that potentially modulate the human immune system requires the examination of multiple variables. To assess the immunotoxic effects in humans exposed to chemical agents, one of the most used approaches is the determination of lymphocyte phenotypic subsets. This includes the quantification of $CD3^+$ (total T cells), $CD4^+$ (T-helper cells), $CD8^+$ (T-cytotoxic cells), $CD19^+$ (B cells) and $CD16^+56^+$ (natural killer cells) subsets. The results obtained from this simple panel are sufficient to display the balance among lymphocyte subpopulations and highlight possible alterations (Colosio *et al.*, 1999).

Lymphocytes are the primary cells involved in acquired immunity and their number may vary from 20 to 40% of the total cells in human blood. They are highly specialised cells that interact with other cells (e.g. macrophage, dendritic cells) to initiate an immune response. The receptor specificity and functional heterogeneity allow them to respond to virtually any antigen (Descotes, 2004; Tryphonas *et al.*, 2005). Furthermore, antigen-specific response results in cell differentiation into memory cells, which remain in the organism and enables a quick response to further insults of that specific antigen, and in effector cells that act by destroying or inactivating the antigen.

Lymphocytes are divided in T and B cells depending on whether cell maturation occurred in the thymus (T) or bone marrow (B).

The use of monoclonal antibodies reacting with CD markers on the lymphocyte surface associated with flow cytometry, allows the identification of T and B cells subpopulations.

CD3 marker along with TCR is expressed on the cell surface of all T lymphocytes (or T cells). These cells are engaged in cell-mediated immune response. T cells are divided into two subpopulations, $CD4^+$ and $CD8^+$, differentiated by their surface marker and effector function. Presence of $CD4^+$ allows the identification of T-helper (Th) cells while $CD8^+$ expression indicates T-cytotoxic lymphocytes (Tc).

These two types of T cells also differ in the class of MHC molecule they recognise; CD4 binds to the MHC class II molecule and CD8 to the MHC class I molecule. MHC class II is only expressed in the cell surface of antigen-presenting cells (e.g. dendritic cells) whereas MHC class I is present on the cell surface of all nucleated cells. During antigen recognition, the TCR associated to either CD4 or CD8 molecules specifically binds to MHC/antigen complex, which activates T-cells leading to an effective response depending on the type of T-cell (Janeway *et al.*, 2001).

CD19 marker is present on the cell surface of all B lymphocytes (or B cells). B cells are precursors of antibody-secreting plasma cells and therefore are involved in the humoral immune response. These cells are activated directly when an antigen specifically binds to a surface immunoglobulin receptor (B-cell receptor) expressed on the cell membrane or indirectly by interaction with T-helper cells, through MHC II B cell, since they are also antigen-presenting cells. Upon activation, B cells differentiate into effector-B cells, called plasma cells, producing antibody molecules of the same antigen specificity as this receptor (Janeway *et al*., 2001).

T cells and B cells play different roles in the adaptive immune response. Broadly, when a foreign substance enters the organism, it is recognised by professional antigen-presenting cells (e.g. dendritic cells) that internalise, degrade and present them as antigens in their surface bound to MHC II. T-helper cells identify that specific antigen in the surface of the antigen-presenting cells, which, as described above, trigger the Th cells to proliferate and differentiate into effector-Th cells. Effector-Th cells main function is to assist B cells activation by bounding to their antigen/MHCII complex or releasing cytokines stimulating cell proliferation. In turn activated effector-B cells (or plasma cells) produce and release antibodies in response to the antigenic attack; their function is to destroy (by tagging for phagocyte action) or inactivate the stimulating antigen (Janeway *et al*., 2001).

T-cytotoxic (Tc) lymphocytes function is to destroy compromised or stressed cells, namely infected cells or tumour cells, by recognition on the cell surface of a specific antigen bound to MHC class I molecule (Janeway *et al*., 2001). Effector-Tc cell capture the target cell through mechanisms of adhesion and release the contents of their granules into it.

This induces apoptosis or disruption of the membrane and the death of the target cell (Abbas and Lichtman, 2004).

A third class of lymphocytes involved in immune responses is natural killer (NK) cells. These cells are closely related to T cells, lacking their receptor and expressing CD16 and CD56 markers on their surface (Tryphonas *et al*., 2005).

NK cells are effector lymphocytes of the innate immune system with cytotoxic and cytokine-production functions. The main role of NK cells is to kill compromised target cells, such as virus-infected cells or tumour cells (Vivier *et al*., 2008). NK cells are able to detect either antibody-coated target cells and exert antibody-dependent cell cytotoxicity by releasing cytotoxic mediators (Descotes , 2004) or “missing self” cells deficient in surface MHC I (Montaldo *et al*., 2013). In fact, the ability to recognise “missing self” cells underlies the mechanism by which NK cells are able to kill stressed cells, such as tumour cells, while sparing normal cells (Vivier *et al*., 2008). Furthermore, it is believed that NK cells are also regulatory cells engaged in reciprocal interactions with dendritic cells, macrophages, T cells and endothelial cells (Vivier *et al*., 2008).

The importance of lymphocyte subpopulations assessment in human biomonitoring is highlighted by several studies linking the exposure to certain substances with immunotoxic properties with altered lymphocytes subsets and their effect on the immune response (Biró *et al*., 2002; Boscolo *et al*., 1999, Oh *et al*., 2005; Tulinska *et al*., 2004).

Furthermore, it was recently demonstrated that an absent, increased or diminished function of immune system cells is clearly associated with autoimmune diseases, deregulation of the immune response, and defective immune response against neoplastic cells or different pathogens (Hernandez- Castro *et al*., 2009). Nevertheless, it should be carefully added that an immunotoxic effect does not always imply clinical consequences (Colosio *et al.*, 1999).

4.2 MATERIAL AND METHODS

4.2.1 Lymphocytes culture

For each cytogenetic assay in lymphocytes, aliquots of heparinised whole blood were cultured in 4.5 mL Hams F10 medium supplemented with 24% foetal bovine serum (FBS, Sigma), penicillin (100 IU/mL) (Sigma), streptomycin (100 µg/mL) (Sigma), 1% L-glutamine (Sigma) and 1% heparin (50 IU/mL) (B. Braun). All cultures were established in duplicate. Lymphocytes were stimulated using 2% (v/v) of phytohaemaglutinin (Gibco BRL) and incubated at 37 ºC.

4.2.2 Chromosomal Aberrations (CAs) Assay

Aliquots of 0.5 mL of heparinised whole blood were cultured as described in 4.2.1. Each culture was established in duplicate and incubated at 37 ºC for 48h. To arrest cells at metaphase stage, demecolcine (Sigma) was added (0.11µg/mL) 3h before the cell harvest. Cells were collected by centrifugation, submitted to hypotonic treatment (KCl 0.075 mol/L), 37 °C for 10 min, fixed twice with ice-cold methanol:acetic acid (3:1, v/v) freshly prepared (454x*g*, 5 min), dropped on coded slides and stained with 4% Giemsa (Merck) in phosphate buffer (PBS) (pH 6.8, Merck) for 10 min. Stained slides were then covered with coverslips and mounted with Entellan®.

CAs analyses were performed on a Nikon Eclipse E400 light microscope, slides were scored blindly by the same reader. One hundred metaphases with well-spread chromosomes were analysed for each individual, fifty from each culture duplicate, using a 500x and 1250x magnification. Gaps, chromosome-type aberrations (CSAs; e.g. chromosomal-type breaks and dicentric and ring chromosomes) and chromatid-type aberrations (CTAs; e.g. chromatid-type breaks, symmetrical homologous figures, radial figures) were identified and classified according to Savage *et al.* (1975). Gaps were not included in CAs parameters. The criteria for distinguishing chromatid-type breaks from gaps were the acentric piece displaced with respect to the chromosome axis and the size of the discontinuity exceeded the width of the chromatid. Acentric fragments were considered together with chromatid-type breaks. A dicentric with an acentric fragment was scored as one aberration. Numerical CAs was also scored and the number of aneuploid metaphases assessed (46 ± 1). Some of the CAs scored are collected in Figure 13.

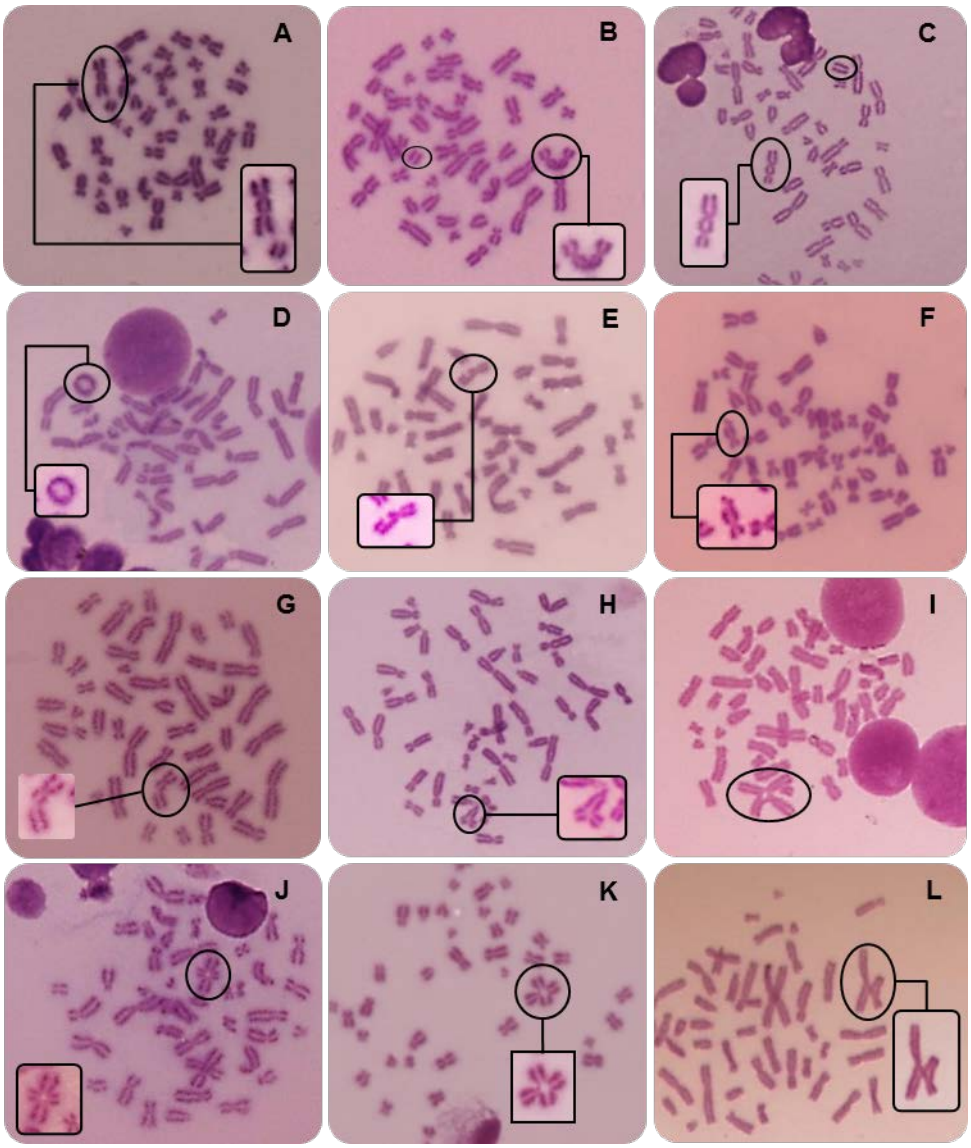


Figure 13. Chromosomal aberrations observed in metaphase (1250x). **A-C)** dicentric chromosome with accompanying fragment; D) ring chromosome; **E-G)** break in one chromatid; **H)** tetraradial figure; **I-K)** symmetrical homologous figures and **L)** symmetrical interchange figure.

In addition, known variables of chromosome fragility were also evaluated for each subject namely the number of aberrant and multiaberrant cells according to Castella *et al*. (2011) and Oostra *et al*. (2012). Exchanges (figures, dicentric and ring chromosomes) were converted into the number of breaks necessary to form each figure, two breaks. A metaphase exhibiting one break (corresponding to a chromatidic-break or a chromosomic-break) was considered aberrant. A metaphase presenting exhanges or two or more fragments or breaks in different chromosomes were accounted as multiaberrant.

4.2.3 **Lymphocyte Cytokinesis-Block Micronucleus Test (CBMN)**

The CBMN test was performed as described elsewhere (Teixeira *et al*., 2004). Briefly, aliquots of 0.5 mL of heparinised whole blood were used to establish duplicate lymphocyte cultures as outlined in 4.2.1. Cytochalasin B (Sigma), a cell-division inhibitor, was added after 44 h at a final concentration of 6 µg/mL. After a total of 72-h culture, cells were collected by centrifugation and treated twice with a mixture (pH 7.2) of RPMI 1640 (Sigma) supplemented with 2% FBS (202x*g*, 7 min). Cells were then submitted to a mild hypotonic treatment in a mixture (pH 7.2) of RPMI:deionised water (1:4, v/v), supplemented with 2% FBS (202x*g*, 5 min). After centrifugation, a drop of the pellet was placed on dry slides and smears were performed. Air-dried slides were fixed (-20 ºC, 20 min) with freshly prepared ice-cold methanol:acetic acid (3:1, v/v) and dried overnight. Slides were stained with 4% Giemsa in pH 6.8 PBS. Stained slides were then covered with coverslips and mounted with Entellan®. Microscopic analyses were performed on a Nikon Eclipse E400 light microscope. To determine the total number of micronucleus in binucleated (BN) cells, a total of 1000 BN cells (500 from each culture duplicate) with well-preserved cytoplasm were scored for each subject. The % of mononucleated, binucleated, trinucleated and tetranucleated cells in 1000 cells was also registered. Micronucleus were scored blindly by the same reader and identified according to the criteria defined by Caria *et al.* (1995) and Fenech (2007) using a 500x and 1250x magnification. Figure 14 are showed some of the images of cells scored in the CBMN assay.

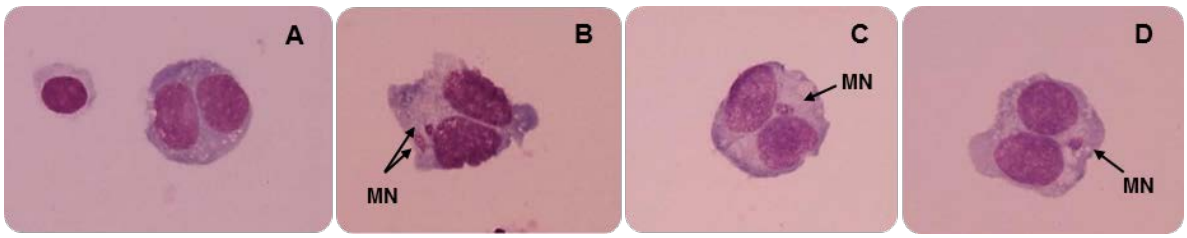


Figure 14. Scored micronucleus (MN) images (500x). **A)** mononucleated and binucleated cells; and **B-D)** binucleated cells with micronucleus.

4.2.4 **Buccal Micronucleus Cytome Assay** (**BMCyt)**

BMCyt was performed as described by Thomas *et al.* (2009) with few modifications. Buccal sampling was performed separately inside of both cheeks, left and right, to eliminate any unknown bias that may be caused by sampling one cheek only. Samples collected were individually suspended in buccal cell buffer (0.01M Tris-HCl, 0.1M EDTA, 0.02 NaCl, pH7). After cells centrifugation, supernatant was removed and replaced with fresh buffer and washed again two more times (728x*g*, 7 min). After the last wash, cells

were resuspended in the small volume of pellet and placed into clean coded slides (3 slides for each cheek). After air-drying, slides were fixed (-20 ºC, 20 min) with cold absolute ethanol: acetic acid (3:1, v/v) freshly prepared. Air-dried fixed slides were treated in 5M HCl for 30 min and washed in running tap water for 3 min. Slides were then stained with Schiff's reagent (Merck) at room temperature in the dark for 60-150 min. Stained slides were washed in running tap water for 5 min and 1 min in deionised water and left to dry in the dark, for 10 min. Slides were then counterstained for 5 sec in 1% Fast Green (Merck) solution, washed in absolute ethanol (3 times, 2 min each) and allowed to air dry. Afterwards, slides were covered with coverslips and mounted with Entellan®. Slides were scored blindly by the same reader on a Nikon Eclipse E400 light microscope using a 500x and 1250x magnification. For each subject the percentage of basal cells, differentiated cells, BN cells and cell death parameters such as condensed chromatin, karyorrhectic, pyknotic and karyolitic cells were scored in 1000 cells. Differentiated cells were scored for micronuclei, nuclear buds and nucleoplasmic bridges. Cells containing micronuclei were confirmed under fluorescence to eliminate false positives. The scoring criteria were based in Tolbert *et al.* (1992) and Thomas *et al.* (2009).

4.2.5 **Sister Chromatid Exchange (SCE) Test**

Aliquots of 0.5 mL of heparinised whole blood were cultured, in duplicate, as described above (4.2.1.) and 5 µL of 5-bromo-2´-deoxyuridine (BrdU, Sigma) were added into the culture medium at a final concentration of 10 µg/mL. Lymphocytes were incubated at 37 ºC in the dark. At 56 h, demecolcine was added (0.11 µg/mL), to arrest cells at metaphase stage, and the cells were incubated for more 1h. Afterwards cells were submitted to a hypotonic treatment (KCl 0.075 mol/L) at 37°C for 10 min and fixed twice with ice-cold methanol:acetic acid (3:1, v/v) freshly prepared (454x*g*, 5 min). Cells were ressuspended in the small volume of pellet and dropped onto clean coded slides. Slides were stained with fluorescence-plus-Giemsa procedure (Perry and Wolff, 1974) for differential chromatid staining. Stained slides were then covered with coverslips and mounted with Entellan®. Microscopic analyses were performed on a Nikon Eclipse E400 light microscope using a 500x and 1250x magnification. A single observer scored a total of 50 well-spread second division metaphases for each subject (half from each duplicate) to determine the number of SCE per cell. Every switch of staining between the sister chromatids was scored as an SCE (Figure 15).

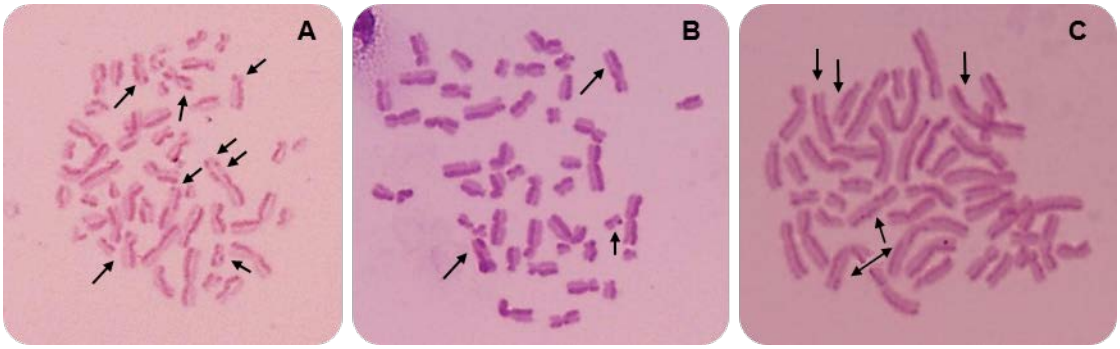


Figure 15. Images of cell metaphases scored for SCE (indicated by arrows) test (1250x).

4.2.6 **Comet assay**

Peripheral blood mononuclear cells were isolated using BD Vacutainer™ CPT™ Cell Preparation Tubes with sodium heparin (Becton Dickinson), following manufacturer's instructions. Cell viability, determined by trypan blue exclusion, was higher than 85% in all cases.

The alkaline comet assay was performed as described by Singh *et al*. (1988) with minor modifications. Briefly, cells collected by centrifugation (4990xg for 3 min) and suspended in 100 µL of 0.6% low-melting-point agarose (LMA) in PBS (pH 7.4) were dropped onto a frosted slide precoated with a 1% layer of normal melting point agarose. Slides were placed on ice for 4 min and allowed to solidify. Coverslips were then removed and slides were immersed in freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 0.25 M NaOH, pH 10; 1% Triton X100) for 1 h at 4 °C, in the dark. After lysis, slides were placed on a horizontal electrophoresis tank in an ice bath. The tank was filled with freshly made alkaline electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) and left in the dark for 20 min to allow DNA unwinding. Electrophoresis was carried out for 20 min at 30 V and 300 mA (1.2 V/cm). Slides were then washed for 10 min with cold neutralizing solution (0.4 M TrisBase, pH 7.5) and 10 min with cold deionised water. After neutralisation, slides were left to air-dry in the dark, overnight. Gels were re-hydrated for 20 min with cold deionised water, stained with ethidium bromide solution (20 µg/mL), washed again for 20 min with cold deionised water and left in the dark for air-drying. For scoring 1 to 2 drops of water were dropped onto each gel and covered with coverslips. Two gels were prepared for each donor and a 'blind' scorer examined 50 randomly cells from each gel (100 cells/donor) using a magnification of x 400. Microscopic analyses were performed on a Nikon Eclipse E400 Epi-fluorescence microscope (G2A filter, Nikon C-SH61). Image capture and analysis were performed with Comet Assay IV software (Perceptive Instruments). The percentage of DNA in the comet tail (%TDNA) was the DNA

damage parameter evaluated. A mix of lymphocytes preserved at -80 ºC, was introduced as an internal standard, the percentage coefficient variation (%CV) obtained was less than 15%.

4.2.7 **TCR mutation assay**

Peripheral blood mononuclear leukocytes were isolated in BD Vacutainer™ Cell Preparation Tubes (CPT™) with sodium heparin according to manufacturer's instructions. After centrifugation, cells were washed three times with ice-cold pH 7.4 phosphate buffer solution. TCR mutation assay was performed by a flow cytometric methodology following Akiyama *et al*. (1995) with minor modifications (García-Lestón *et al*., 2011). Cell suspensions were analysed by a FACScalibur flow cytometer with *Cell Quest Pro* software (Becton Dickinson). A minimum of 2.5×10^5 lymphocyte-gated events were acquired, and mutation frequencies of TCR (TCR-Mf) were calculated as the number of events in the mutant cell window ($CD3^-CD4^+$ cells) divided by the total number of events corresponding to $CD4^+$ cells.

4.2.8 **Lymphocyte subpopulations assessment**

Cell percentages of total T lymphocytes (%$CD3^+$), T-helper (Th) lymphocytes (%$CD4^+$), T-cytotoxic (Tc) lymphocytes (%$CD8^+$), B lymphocytes (%$CD19^+$) and natural killer (NK) cells (%$CD16^+56^+$) were determined by flow cytometric measurements using a three-color direct immunofluorescence surface marker methodology, as described by García-Lestón *et al*. (2011). The following antibodies were used: fluorescein isothiocyanate (FITC)-labelled antiCD3, phycoerytrin (PE)-labelled antiCD4, phycoerytrin-cyanin 5 (PE-Cy5)-labelled antiCD8, PECy5-labelled antiCD19, and PE-labelled CD16 and CD56. Analyses were carried out in a FACScalibur flow cytometer using *Cell Quest Pro* software (Becton Dickinson). After gating the lymphocytes based on forward/side scatter plots, fluorescence data from FL1 (FITC), FL2 (PE) and FL3 (PE-Cy5) were obtained. At least 10^4 events in the lymphocytes window were acquired.

4.3 RESULTS

4.3.1 Biomarkers of genotoxicity

Univariate comparisons of effect biomarkers by study group are reported in Table VI. All genotoxicity endpoints, with the exception of TCR-Mf, were significantly increased in the FA-exposed workers compared to control subjects.

Table VI. Results of biomarkers of genotoxicity in the study groups.

	Controls		Exposed		
	N	**mean ± SE** *(range)*	***N***	**mean ± SE** *(range)*	***p*-value**
CA-total	*87*	2.09 ± 0.25 *(0-13)*	*84*	3.96 ± 0.34 *(0-13)*	**<0.001**
CSAs CA-chromosome type	*87*	0.48 ± 0.10 *(0-4)*	*84*	0.98 ± 0.14 *(0-5)*	**0.004**
CTAs CA-chromatid type	*87*	1.61 ± 0.19 *(0-10)*	*84*	3.00 ± 0.28 *(0-12)*	**<0.001**
Gaps	*87*	3.49 ± 0.32 *(0-14)*	*84*	5.70 ± 0.31 *(0-13)*	**<0.001**
Aneuploidies	*87*	2.13 ± 0.19 *(0-6)*	*84*	3.49 ± 0.19 *(0-8)*	**<0.001**
MNL (‰) MN in lymphocytes	*87*	2.79 ± 0.30 *(0-10)*	*84*	4.38 ± 0.38 *(0-18)*	**<0.001**
MNB (‰) MN in buccal cells	*69*	0.17 ± 0.05 *(0-2)*	*63*	0.65 ± 0.09 *(0-4)*	**<0.001**
BNbud (‰) Nbud in buccal cells	*69*	0.32 ± 0.06 *(0-2)*	*63*	0.94 ± 0.11 *(0-3)*	**<0.001**
SCE/cell	*87*	4.01 ± 0.11 *(1.82-6.16)*	*84*	5.08 ± 0.12 *(2.00-8.78)*	**<0.001**
%TDNA Comet assay	*87*	7.50 ± 0.47 *(0.86-*24.40)	*83*	11.67 ± 0.72 *(0.23-28.07)*	**<0.001**
TCR-Mf ($x10^{-4}$) TCR mutation frequency	*64*	3.69 ± 0.43 *(1.08-27.95)*	*61*	3.90 ± 0.45 *(1.28-26.29)*	0.733

With regard to CAs types, both CSAs (chromosome-type aberrations) and CTAs (chromatid-type aberrations) were significantly higher in FA-exposed workers compared to controls. The latter was the most frequent type of aberration found in both groups, which is consistent to the fact that most of spontaneous aberrations are of chromatid-type, although with low frequency (Savage, 1975). In addition, other parameters regarding the CAs were assessed namely the frequency of aberrant (with one break) and multiaberrant cells (with two or more breaks). The results are showed in Table VII. Both variables were significantly higher in the exposed group than in the control group.

Table VII. Results of aberrant cells and multiaberrant cells in the study groups

	Controls		Exposed		
	N	**mean ± SE** *(range)*	***N***	**mean ± SE** *(range)*	***p*-value**
Aberrant cells	*87*	1.90 ± 0.19 *(0-9)*	*84*	3.18 ± 0.28 *(0-11)*	**0.001**[a]
Multiaberrant cells	*87*	0.14 ± 0.04 *(0-2)*	*84*	0.55 ± 0.09 *(0-3)*	**<0.001**[a]

4.3.1.1 ***Effect of exposure, lifestyle factors and parameter-specific confounders***

To evaluate the genotoxic effect of exposure and the possible influence of some known confounders on the frequencies of the endpoints studied a multivariate modelling was carried out. All models included gender, age and smoking habits. A parameter-specific confounder was found for %TDNA, fruit consumption, and therefore it was included on the analysis. The influence of the level of formic acid in urine was also evaluated, however no significant effect was found in any of the considered biomarkers. Consequently, this variable was not included on the final statistical models. The result on genotoxicity biomarkers, adjusted for gender, age, smoking habits and actual parameter-specific confounders, are reported in Table VIII, Table IX and Table X.

The significant effect of exposure was confirmed in all the cytogenetic biomarkers studied (CA-total, CSAs, CTAs, gaps, aneuploidies, MNL, MNB, BNbud and SCE; Tables VIII and IX) and in the DNA damage parameter (%TDNA; Table X) with FA-exposed workers showing increased frequencies compared to controls. No significant influence of gender was observed on the frequencies of the endpoints studied.

Age was found to be a significant confounder but only for CSAs, MNL and MNB. CSAs frequency was significantly increased in subjects between ages 35-45 (Table VIII). It was also elevated in individuals above 45 years-old, but with a near significance (p= 0.06). Concerning MNL and MNB, both endpoint frequencies were significantly higher in subjects above 35 years old (Tables IX). Indeed, a significant positive correlation was found between age and MNL frequency (r=0.287, p<0.001) and MNB frequency (r=0.319, p<0.001).

With regard to smoking habits, a significant effect was found for aneuploidies (decrease) and MNB (increase) frequencies. Significant associations found between these variables and packs/year further confirmed the influence of smoking on the frequencies of these endpoints. A negative correlation with aneuploidies (r=-0.270, p<0.001) and a positive with MNB (r=0.195, p=0.025).

Table VIII. Effect of exposure, gender, age and smoking habits on the frequencies of CA-total, CSAs, CTAs, gaps and aneuploidies with estimates of mean ratios (MR)

		CA-total		CSAs		CTAs		Gaps		Aneuploidies	
	N	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
Exposure											
Controls	85	1.00		1.00		1.00		1.00		1.00	
Exposed	87	1.91***	[1.44-2.53]	2.07**	[1.27-3.38]	1.86***	[1.39-2.48]	1.65***	[1.34-2.03]	1.64***	[1.36-1.98]
Gender											
Females	133	1.00		1.00		1.00		1.00		1.00	
Males	39	0.85	[0.60-1.21]	0.53	[0.27-1.05]	0.98	[0.68-1.42]	1.03	[0.80-1.33]	0.89	[0.70-1.13]
Age											
<35 years	60	1.00		1.00		1.00		1.00		1.00	
35-45 years	63	1.11	[0.80-1.55]	1.89*	[1.03-3.46]	0.97	[0.69-1.37]	0.98	[0.77-1.24]	0.89	[0.71-1.11]
>45 years	49	1.20	[0.84-1.70]	1.84	[0.98-3.46]	1.08	[0.76-1.54]	0.96	[0.75-1.25]	0.99	[0.79-1.24]
Smoking Habits											
Non-smokers	129	1.00		1.00		1.00		1.00		1.00	
Smokers	43	0.82	[0.58-1.15]	0.88	[0.48-1.60]	0.79	[0.56-1.14]	0.81	[0.63-1.05]	0.67**	[0.52-0.86]

* p<0.05
** p<0.01
*** p<0.001

Table IX. Effect of exposure, gender, age and smoking habits on the frequencies of MNL, SCE, MNB and BNbud with estimates of mean ratios (MR)

		MNL (‰)		**SCE**/cell			**MNB** (‰)		**BNbud** (‰)	
	N	**MR**	**[95% CI]**	**MR**	**[95% CI]**	**N**	**MR**	**[95% CI]**	**MR**	**[95% CI]**
Exposure										
Controls	85	1.00		1.00		69	1.00		1.00	
Exposed	87	1.55**	[1.20-1.99]	1.27**	[1.10-1.46]	63	4.08***	[2.12-7.87]	2.88***	[1.76-4.71]
Gender										
Females	133	1.00		1.00		100	1.00		1.00	
Males	39	1.07	[0.77-1.48]	1.06	[0.89-1.26]	32	1.41	[0.76-2.63]	1.02	[0.58-1.76]
Age										
< 35 years	60	1.00		1.00		49	1.00		1.00	
35-45 years	63	1.40*	[1.03-1.91]	1.11	[0.94-1.32]	47	2.32*	[1.05-5.11]	1.00	[0.57-1.75]
> 45 years	49	1.97***	[1.44-2.71]	1.10	[0.92-1.31]	36	3.54**	[1.64-7.66]	1.61	[0.95-2.73]
Smoking Habits										
Non-smokers	129	1.00		1.00		94	1.00		1.00	
Smokers	43	0.88	[0.64-1.20]	1.13	[0.96-1.34]	38	2.12**	[1.22-3.69]	0.87	[0.52-1.46]

* p<0.05
** p<0.01
*** p<0.001

Table X. Effect of exposure, gender, age and smoking habits on the frequencies of %TDNA and TCR-Mf with estimates of mean ratios (MR)

	%TDNA			TCR-Mf ($x10^{-4}$)		
	N	**MR**	**[95% CI]**	**N**	**MR**	**[95% CI]**
Exposure						
Controls	85	1.00		69	1.00	
Exposed	87	1.50**	[1.14-1.96]	61	1.03	[0.74-1.43]
Gender						
Females	133	1.00		98	1.00	
Males	39	1.05	[0.75-1.47]	32	1.20	[0.81-1.77]
Age						
< 35 years	60	1.00		48	1.00	
35-45 years	63	1.22	[0.88-1.67]	47	0.96	[0.65-1.42]
> 45 years	49	1.10	[0.78-1.56]	35	1.02	[0.68-1.54]
Smoking Habits						
Non-smokers	129	1.00		93	1.00	
Smokers	43	0.88	[0.64-1.20]	37	0.87	[0.59-1.28]
Fruit consumption (no. pieces/day)	130	0.88*	[0.78-0.99]	-	-	-

***** p<0.05
****** p<0.01

In addition, fruit consumption was found to significantly decrease the DNA damage evaluated by comet assay (%TDNA) (Table X). Regarding aberrant and multiaberrant cells parameters no significant effect was obtained for gender, age or smoking habits (data not showed).

4.3.2 **Immune Markers: Lymphocytes subpopulations**

Data obtained for univariant analysis of lymphocyte subpopulations in FA-exposed subjects and controls are reported in Table XI. Statistically significant differences were found for $\%CD8^+$ (T-cytotoxic cells), $\%CD19^+$ (B lymphocytes) and $\%CD16^+56^+$ (NK cells). The results found for $CD4^+/CD8^+$ ratio (Th/Tc) confirmed the effect on $\%CD8^+$. No significant differences were observed for $\%CD3^+$ (total T lymphocytes) and $\%CD4^+$ (T-helper cells).

Table XI. Results of lymphocyte subpopulations in the study groups.

	Controls		Exposed		
	N	**mean ± SE** *(range)*	***N***	**mean ± SE** *(range)*	***p*-value**
Total T lymphocytes $\%CD3^+$	*64*	73.82 ± 0.96 *(49.99-88.6)*	*61*	72.73 ± 1.10 *(46.14-87.42)*	0.455
T-helper cells (Th) $\%CD4^+$	*64*	44.54 ± 1.10 *(26.35-73.2)*	*61*	45.03 ± 1.08 *(23.76-63.48)*	0.754
T-cytotoxic cells (Tc) $\%CD8^+$	*64*	16.85 ± 0.90 *(5.49-39.33)*	*61*	24.89 ± 1.11 *(10.79-50.71)*	**<0.001**
B cells $\%CD19^+$	*64*	23.31 ± 1.13 *(4.64-44.4)*	*61*	11.30 ± 0.47 (3.02-20.08)	**<0.001**
Natural killer (NK) cells $\%CD16^+56^+$	*64*	9.95 ± 0.78 *(1.66-39.17)*	*61*	12.56 ± 0.95 *(1.92-44.59)*	**0.035**
Th /Tc ratio $CD4^+/CD8^+$ ratio	*64*	3.34 ± 0.25 *(1.04-8.74)*	*61*	2.10 ± 0.13 *(0.47-5.2)*	**<0.001**

4.3.2.1 ***Effect of exposure, lifestyle factors and parameter-specific confounders***

Table XII summarises the results obtained in the multivariant analysis of the immune markers taking into account gender, age and smoking habits in addition to exposure. The analysis was also adjusted for actual parameter-specific confounders, namely vitamin supplement intake for T-cytotoxic cells and fruit consumption for T-helper cells. Formic acid in urine was not included in the final multivariate models, since it proved not to be a modifying factor on the lymphocyte subsets.

Significant effect of exposure were confirmed for percentages of T-cytotoxic cells (increase), B lymphocytes (decrease), NK cells (increase) and Th/Tc ratio (decrease).

Table XII. Effect of FA-exposure, gender, age and smoking habits on lymphocyte subpopulations with estimates of mean ratios (MR)

		Total T lymphocytes %CD3$^+$		**T-helper cells** %CD4$^+$		**T-cytotoxic cells** %CD8$^+$		**B cells** %CD19$^+$		**NK cells** %CD16$^+$56$^+$		**Th/Tc** CD4+ /CD8$^+$	
	N	**MR**	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**
Exposure													
Controls	64	1.00		1.00		1.00		1.00		1.00		1.00	
Exposed	61	0.98	[0.94-1.03]	1.02	[0.96-1.09]	1.47**	[1.29-1.68]	0.51**	[0.44-0.59]	1.30*	[1.02-1.64]	0.69**	[0.59-0.82]
Gender													
Females	95	1.00		1.00		1.00		1.00		1.00		1.00	
Males	30	1.01	[0.96-1.06]	1.01	[0.93-1.10]	0.81*	[0.69-0.96]	1.11	[0.92-1.34]	1.20	[0.90-1.61]	1.22	[1.00-1.50]
Age													
< 35 years	43	1.00		1.00		1.00		1.00		1.00		1.00	
35-45 years	47	1.08**	[1.03-1.13]	1.06	[0.98-1.14]	1.07	[0.92-1.26]	0.84	[0.70-1.00]	0.85	[0.64-1.12]	0.98	[0.81-1.20]
> 45 years	35	1.06*	[1.01-1.12]	1.19**	[1.10-1.30]	0.77**	[0.65-0.91]	0.90	[0.74-1.08]	0.82	[0.61-1.10]	1.56**	[1.26-1.93]
Smoking Habits													
Non-smokers	90	1.00		1.00		1.00		1.00		1.00		1.00	
Smokers	35	1.01	[0.97-1.06]	1.04	[0.96-1.12]	0.95	[0.81-1.11]	1.02	[0.86-1.22]	1.05	[0.79-1.38]	1.11	[0.91-1.34]
Fruit consumption (no. pieces/day)	125	–	–	1.03	[1.00-1.06]	–	–	–	–	–	–	–	–
Vitamin suppl. intake (Yes/No)	125	–	–	–	–	1.30*	[1.05-1.60]	–	–	–	–	–	–

*p<0.05
**p<0.01

Regarding gender, males had a significantly lower percentage of T-cytotoxic cells compared with females.

Furthermore, age significantly influenced total T lymphocyte, T-helper cells and T-cytotoxic cells subsets. Total T lymphocytes were higher in subjects above 35 years-old while T-helper cells were also significantly increased but only in individuals above age 45. A contrasting effect in the T-cytotoxic cells subset was found for that group age (> 45 years-old) with a significant decrease. The effect of age in these endpoints was further confirmed by correlation analysis. Indeed, a significant positive association was found for Total T lymphocytes ($r=0.259$, $p=0.004$) and T-helper cells ($r=0.375$, $p<0.001$), whereas for T-cytotoxic cells a negative association ($r=-0.192$, $p=0.032$) was found with age.

No significant effect of smoking status was observed on lymphocyte subpopulations.

Regarding actual parameter-specific confounders, vitamin supplement intake was found to significantly increase T-cytotoxic cells and fruit consumption was found to have a near significant increase on % T-helper cells ($p=0.05$).

4.3.3 **Effect of work-related factors**

In the exposed population the influence of FA-level of exposure corresponding to each exposed individual, time of exposure and professional activity on genotoxicity and immune markers was also evaluated by multivariate regression, results are described in Table XIII (only parameters for which at least one significant result was obtained are shown).

With regard to genotoxicity biomarkers, MNL and BNbud frequencies were significantly increased with FA exposure level. Concerning lymphocyte subpopulations, NK cells (%$CD16^{+}56^{+}$) were significantly decreased with the FA-level of exposure. This effect was further confirmed with a nearly significant association between %NK cells and FA exposure level ($r= -0.245$, $p=0.054$).

No significant effects were obtained with exposure duration or professional activities in any of the endpoints studied. However, a positive significant correlation was found between MNL frequencies and exposure duration ($r=0.277$, $p=0.011$).

To better investigate the effect of the estimated FA-levels of exposure on the frequencies of MNL, BNbud and %NK cells, the variable was categorised into tertiles: T1, ranged 0.08-0.22 ppm; T2, ranged 0.23-0.34 ppm; and T3 ranged 0.35-1.39 ppm, results are shown in Table XIV. Subjects in the categories T2 and T3, with higher exposure levels, had a significant increase in MNL frequency compared to reference category T1 (lowest exposure).

Table XIII. Influence of FA-exposure level, exposure duration and professional activity on micronuclei in lymphocytes (MNL), nuclear buds in buccal cells (BNbud) and %$CD16^{+}56^{+}$ cells, with estimates of mean ratios (MR)

	MNL (‰)			**BNbud** (‰)			**%$CD16^{+}56^{+}$**		
	N	**MR**	**[95% CI]**	**N**	**MR**	**[95% CI]**	**N**	**MR**	**[95% CI]**
FA-level of exposure (ppm)	84	2.71*	[1.18-6.23]	63	5.27*	[1.27-21.90]	61	0.32*	[0.12-0.84]
Exposure duration									
<8 years	28	1.00		25	1.00		24	1.00	
8-14 years	28	0.78	[0.51-1.23]	18	0.74	[0.30-1.78]	17	1.00	[0.63-1.60]
>14 years	28	0.68	[0.40-1.15]	20	1.00	[0.37-2.74]	20	1.31	[0.75-2.28]
Professional activity									
Physician	30	1.00		23	1.00		21	1.00	
Technician	40	0.91	[0.61-1.35]	30	1.90	[0.94-3.86]	30	0.92	[0.60-1.41]
Hospital orderlies	10	0.78	[0.43-1.42]	7	1.07	[0.35-3.20]	7	1.76	[0.96-3.23]
Administrative	4	0.95	[0.44-2.07]	3	-	-	3	0.89	[0.39-2.03]

*p<0.05

Table XIV. Influence of FA-exposure level of exposure categorized by tertiles on micronuclei in lymphocytes (MNL), nuclear buds in buccal cells (BNbud) and %$CD16^{+}56^{+}$ cells, with estimates of mean ratios (MR)

	MNL (‰)			**BNbud** (‰)			**%$CD16^{+}56^{+}$**		
	N	**MR**	**[95% CI]**	**N**	**MR**	**[95% CI]**	**N**	**MR**	**[95% CI]**
FA-level of exposure									
T1 (0.08-0.22 ppm)	27	1.00		20	1.00		20	1.00	
T2 (0.23-0.34 ppm)	29	1.50**	[1.12-2.00]	16	1.42	[0.64-3.14]	25	1.05	[0.69-1.60]
T3 (0.35-1.39 ppm)	28	1.37*	[1.04-1.81]	17	1.96	[0.91-4.24]	16	0.50**	[0.32-0.79]

*p<0.05
**p<0.01

The same effect was observed for BNbud, but it did not reach significance. For %NK cells, no significant difference was observed between T1 and T2. In contrast, a marked and significant decrease in %NK cells was found in the highest tertile, T3, corresponding to workers exposed to estimated levels of FA equal or higher than 0.35 ppm. This trend is further confirmed by the mean %NK values found in these three groups: T1, 12.78%; T2, 15.22% and T3, 8.12% (this last value even lower than the observed for the control group, 9.95%).

4.3.4 **Correlations between biomarkers**

The associations between the different studied biomarkers were evaluated and some were found to be significant. As expected, CA-total parameter was significantly well correlated with CSAs (r=0.632 p<0.001), CTAs (r=0.930 p<0.001), aberrant (r=0.953 p<0.001) and multi-aberrant cells (r=0.580 p<0.001). A significant positive association was also found between gaps and CA-total (r=0.521 p<0.001), CSAs (r=0.291 p<0.001), CTAs (r=0.498 p<0.001), aberrant (r=0.511 p<0.001) and multi-aberrant cells (r=0.315 p<0.001). In contrast, no significant correlations were found for aneuploidies and TCR-Mf with any of the biomarkers. Further associations found between the genotoxicity endpoints are gathered in Table XV.

Table XV. Associations between biomarkers of genotoxicity

Endpoint 1	Endpoint 2	Correlation coefficient (*r*)
CTAs	CSAs	0.343***
Gaps	MNL	0.233**
	%TDNA	0.273***
MNL	Total-CAs	0.299***
	CSAs	0.162*
	CTAs	0.295***
	MNB	0.359***
MNB	BNbud	0.261**
SCE	Total-CAs	0.172*
	CTAs	0.159*
	Gaps	0.156*
%TDNA	CSAs	0.174*

***** p<0.05
****** p<0.01
*** p<0.001

Concerning lymphocyte subpopulations a significant inverse association was found between T-cytotoxic cells and B cells (r=-0.577 p<0.001). Analysis of correlations between

the different biomarkers of genotoxicity and lymphocyte subpopulations showed that these were significant for CA-total and T-cytotoxic cells ($r=0.376$, $p<0.001$) and B cells ($r=-0.313$ $p<0.001$). Similarly, between SCE frequency and T-cytotoxic cells ($r=0.204$ $p<0.05$) and B cells ($r=-0.352$ $p<0.001$). And lastly for %TDNA and T-cytotoxic cells ($r=0.296$ $p<0.001$) and B cells ($r=-0.350$, $p<0.001$).

4.4 DISCUSSION

As observed, occupational exposure to Formaldehyde (FA) is common in pathological anatomy laboratories, where professionals are often exposed to FA-airborne concentrations near or superior to recommended limit values. FA was recently classified carcinogenic to humans. Consisting findings of increased risks of rare cancers, such as nasopharyngeal and sinonasal cancer were found among workers with higher measures of exposure to FA (exposure level or duration).

In addition, an excess of mortality from leukaemia was observed in epidemiological studies involving embalmers, funeral directors and healthcare professionals, namely, pathologists and anatomists. It has been speculated that these findings might be explained by concurrent exposure to other chemicals (Golden, 2011), however none of the compounds used in these settings have established leukaemogenic properties (Hauptmann *et al*., 2009). Moreover, the exposure to other chemicals would differ between professional groups, which reduce the likelihood that such exposures could explain the observed increases in risk (IARC, 2006). Furthermore, in a meta-analysis that included 18 studies, it was reported that the overall increase of relative risk for leukaemia among embalmers, pathologists and anatomists did not vary significantly between studies, and the results were found to be homogeneous (Collins and Lineker, 2004).

The mechanisms by which FA causes cancer are not completely understood but most likely involve multiple modes of action, including genotoxicity by DNA reactivity, chromosomal breakage, aneuploidy or oxidative stress (Zhang *et al.*, 2010a; NTP, 2011).

Although, FA genotoxicity has been demonstrated *in vitro* experiments on cell lines and laboratory animals, data regarding occupationally exposed individuals are often inconsistent and controversial, particularly in what concerns FA-induced genotoxic effects in circulating peripheral blood lymphocytes (PBLs).

4.4.1 Biomarkers of genotoxicity

Genotoxicity evaluation constitutes a valuable tool for studying the most important occupational hazards to public health occurring in the past few decades and allows a reasonable epidemiological evaluation of cancer predictivity (Bonassi *et al.*, 2005; Laffon *et al.*, 2006). Cytogenetic biomarkers such as chromosomal aberrations (CAs), micronucleus (MN) and sister chromatid exchanges (SCE) are well-established endpoints that have been extensively used for assessing DNA damage at the chromosomal level in human biomonitoring studies (Carrano and Natarajan, 1988; Fenech, 1993).

In this study, all cytogenetic parameters evaluated (Total-CA, CSAs, CTAs, gaps, aneuplodies, MNL, MNB, BNbud and SCE) were significantly elevated in pathological anatomy professionals exposed to FA (0.38 ppm) compared with control subjects (Table VI).

In fact, FA-exposed individuals showed an increase of 91% on Total-CAs frequency compared to controls (Table VIII). Furthermore, mean frequencies of both CAs types, CSAs (chromosome-type aberrations) and CTAs (chromatid-type aberrations) were significantly higher in exposed workers than controls (Table VI). Although there is a paucity of studies assessing CAs in FA occupational exposed subjects, our findings are in agreement with most of the published literature. Indeed, increased frequencies of CAs were found in PBLs of anatomy students exposed to FA (mean level, 2.37 ppm) during a 12-week (10h per week) anatomy class (He *et al.*, 1998). Similarly, in a recent study involving FA-exposed personnel working in pathology departments (n=21; mean level=0.72 ppm) Total-CA and CTAs were significantly elevated compared to controls, no significant differences were found for CSAs (Jakab *et al.*, 2010). Moreover, *in vitro* experiments of Schmid *et al.* (1986) with human lymphocytes showed that FA induces CAs, mostly of chromatid-type and gaps, as observed in occupational exposed subjects. A significant increase on CAs frequencies were also observed in industrial workers, namely in a group of paper factory workers, i.e. dicentric and rings chromosomes (Bauchinger and Schmid, 1985), and among workers manufacturing nitrogen fertiliser and exposed to FA concentrations exceeding permissible levels (Kitaeva *et al.*, 1996). In addition, a study in former Czechoslovakia reported increased levels of CAs in PBLs of children exposed to FA in prefabricated schools (Neri *et al.*, 2006).

In opposition, Thomson *et al*. (1984) found no significant differences in the incidences of CAs in a group of six pathology workers compared to unexposed controls. The same result was obtained among FA-exposed medical students working in an anatomy dissection laboratory (Vasudeva and Anand, 1996). Further, no significant differences were found on CAs frequencies between individuals working in different laboratories of a Cancer Research Institute including an anatomical pathology laboratory (Pala *et al.*, 2008), however the results obtained may be attributed to the low level of FA exposure (range, 0.01-0.05 ppm) and the reduced number of subjects evaluated.

In the present study, both CAs sub-types were statistically elevated in PBLs of FA-exposed workers. As referred earlier, the generation of structural CAs requires one or several DNA double-strand breaks (DSBs), CSAs and CTAs formation appear to occur in different cellular phases, involve specific DNA repair mechanisms and induce by different agents. While CSAs are thought to be formed by direct double stranded breakage *in vivo*

in *G0/G1* lymphocytes (being duplicated during replication), CTAs formation requires DNA-replication and arise *in vitro* from other lesions pre-existing in DNA. However, CTAs that survive to an earlier division can be converted by duplication into secondary chromosome-types, and in next cell generation appear as CSAs (Savage, 1975; Musak *et al.*, 2013). Indeed the significant positive association found in our study between CTAs and CSAs ($r=0.343$, $p<0.001$) may confirm this association and explain our results. Nevertheless, it should be noted that mechanistically, for instance, two close single-strand breaks *in vivo*, not repaired in G1, might produce in metaphase a chromosome type break.

Hence, our findings indicates that FA- exposure is able to induce lesions in chromosomal DNA which during repair or DNA synthesis generates DSBs and CAs formation. In addition, some stable forms of CTAs seem to survive division and pass in a modified form to next cell generation.

Other variables were analysed in order to obtain more information about the biological impact of our results. Therefore, we assessed the number of aberrant cells (cells with one CSAs or CTAs break) and multiaberrant cells (cells with CSAs or CTAs type exchanges or with more of two breaks/fragments). Significant differences were obtained in both variables, with FA-exposed subjects showing elevated number of aberrant and multiaberrant cells compared to controls.

While the majority of CAs are lethal to the cell, others may lead to oncogenic transformation, for instance by inactivation of a tumour suppressor gene or activation of an oncogene by generating novel fusion proteins capable of initiating carcinogenesis (Pfeiffer *et al*., 2000). Indeed, accumulated data confirms that high CAs frequencies in lymphocytes of healthy individuals are predictive of cancer risk (Hagmar *et al.*, 1998). A number of factors may collectively influence the association between CAs and cancer including exposure to genotoxic carcinogens and internal generation of genotoxic species (e.g. oxidative stress) (Norppa *et al*., 2006). In fact, a significant association was found between cancer incidence and CAs (chromatid breaks) and aberrant cells frequency in a group of miners exposed to radon – the authors estimated that an increase of 1% in the frequency of CAs was associated with an increased cancer incidence of 64% (Smerhovsky *et al*., 2001, 2002).

Our results also showed that FA-exposed workers had significantly higher frequencies of gaps (a 65% increase) than controls (Table VIII). Schmid *et al*. (1986) and Jakab *et al*. (2010) found similar results *in vitro* experiments and in PBLs of pathologists exposed to FA, respectively. Previous reports of individuals occupationally exposed to other

chemicals also found significant increases on gaps compared to referents (Tunca and Egeli, 1996; Lander *et al*., 2000; Goud *et al*., 2004; Almeida-Santos *et al*., 2005; Skjelbred *et al*., 2011). The validity of scoring and analysing gaps in human biomonitoring studies has been the subject of much discussion. One reason is the visual distinction of a gap from a break. Although a consensus was reach on scoring criteria their inclusion in studies remain controversial (Oostra *et al.*, 2012). Other point of discussion is whether a gap represents a true double-stranded break in the DNA of a chromatid, a staining discontinuity or an error in chromosome condensation process ("folding defect") (Savage, 2004). Our findings, revealed significant positive correlations between gaps and all structural CAs endpoints, showing an association with established CAs parameters, and confirming this parameter sensitivity for FA genotoxicity evaluation. In fact, results from a recent comparative analysis of different types of CAs observed under light microscopy and by means of atomic force microscopy showed that most gaps are chromosome alterations and should be included in genotoxicity studies (Koleva *et al*., 2013). This statement is further supported by evidence that DNA lesions such as DSBs may affect chromatin condensation (Savage, 2004), and by the observation that chromatin relaxation is a fundamental pathway in the DNA-damage response (Ziv *et al*., 2006). Therefore, the increase of gaps in FA-exposed subjects may occur as a response to double-stranded breakage. Also of note, in opposition to CAs, gaps are reparable, and produce no further structural damage on transmission (Savage, 2004), so by being a reversible form of damage it may reflect relatively recent genotoxic exposure (Lander *et al*., 2000). Paz-y-Miño *et al*. (2002) tested the correlation between gaps and comet assay, a biomarker of recent damage, with positive results. Indeed, we also found a significant positive association between gaps and the comet assay parameter ($r=0.273$; $p<0.001$), which confirm the above hypothesis of the possible biological significance of gaps, and reinforces the inclusion of this event as a parameter in biomonitoring studies.

In our study, a significant increase in aneuploid PBLs was found among FA-exposed workers compared to controls (Table VI). However, there are limited data available evaluating the frequency of numerical CAs in occupational subjects exposed to FA and published results are conflicting (Zhang *et al*. 2010b, Speit *et al*., 2011). An earlier study reported an increase in aneuploid cells in a group of pathologists compared to controls, but it did not reach significance (Thomson *et al*., 1984). Conversely, a significant decrease in aneuploidy was found among workers exposed to FA (0.72 ppm) in a pathology department, but the observed lower frequency of aneuploid cells may be attributed to the significant increase of apoptotic cells found in the FA-exposed workers investigated (Jakab *et al.*, 2010). In fact, *in vitro* experiments on cell lines showed that at

concentrations around 1mM, FA enhanced apoptosis and decreased cell proliferation, whereas at lower doses (0.1 mM) it decreased apoptosis and increased cell proliferation (Tyihák *et al*., 2001). Although apoptosis induction was not evaluated in our study, this finding may explain the different outcome found in our group of workers exposed to 0.38 ppm compared to Jakab *et al*. (2010) study (0.72 ppm). Furthermore, in a recent study FA showed to deregulate the expression of miRNAs involved in apoptosis signalling within the nasal rats epithelium exposed to FA (Rager *et al*., 2013), which was in accordance with previous findings in pulmonary cells of rats also exposed to FA (Sul *et al*., 2007).

Other studies based on a different assay (e.g. fluorescence in situ hybridisation) have also yielded mixed results, with cells of professionals exposed to FA showing a significant increase of chromosome loss in one study (Orsière *et al*., 2006) and no significant effect in another (Titenko-Holland *et al*., 1996).

Nevertheless, it should be noted that aneuploidy is a natural event occurring in healthy subjects. Indeed, different studies have showed that in humans about 3% of lymphocytes are aneuploid (Fang and Zhang, 2011). It is possible that aneuploid cells are present in all tissue types and because of their presence in low percentages they do not post any significant pathological danger including oncogenic transformation. On the other hand, aneuploidy is a prominent phenotype of cancer, and it has been discussed whether aneuploidy is only a byproduct of the oncogenic processes or if it can induce tumorigenesis (Fang and Zhang, 2011). Zhang *et al*. (2010b) reported leukaemia-specific chromosome changes (monosomy 7 and trisomy 8) in cultured peripheral blood myeloid progenitor cells of workers exposed to FA, this finding suggest that FA- exposure may have an adverse impact through an aneugenic effect on the hematopoietic system. However, the mechanisms behind aneuploidy are difficult to dissect due to countless factors that may be involved. Therefore, taking in consideration the above mentioned further studies on the FA potential aneugenic activity are needed before reaching to any conclusion.

The results of micronucleus (MN) frequency in PBLs and buccal cells are shown in Table VI. Compared to controls, MN frequency was significantly increased in FA-exposed workers in both PBLs and exfoliated buccal cells. MN frequency in PBLs was found to be approximately 1.6-times higher in the exposed workers compared to control subjects, whereas in buccal cells an around 3.8-fold increase was observed. Furthermore, a significant higher frequency of nuclear buds (BNbud) in exfoliated buccal cells was also found among workers in comparison to controls (2.9-times higher). The increase of

damage was higher in buccal cells, which is not surprising since the oral mucosa constitutes a site of first contact for inhaled FA.

Our findings comply with others that reported an increased frequency of MN in PBLs and/or epithelial cells among mortuary students and, more recently, in hospital staff exposed to FA. Indeed, Suruda *et al*. (1993) found significant increased frequencies of MN in lymphocytes (26%) and buccal mucosa cells (12-fold) in a group of mortician students exposed to FA (8h-TWA level, 0.33 ppm) before and after attending to an embalming course. A dose-response relationship was observed with cumulative exposure, but only for buccal micronuclei. Ying *et al*. (1997) also reported a significant higher frequency of MN in nasal and oral exfoliated cells in anatomy students exposed to FA after an 8-week course (8h-TWA level, 0.41 ppm), but no significant difference was found for lymphocytes. However, a significant elevated frequency in micronucleated lymphocytes was found among 59 pathology and anatomy laboratory workers in a study conducted by Orsière *et al*. (2006). Burgaz *et al*. (2001, 2002) evaluated MN induction in buccal and nasal epithelium from pathology and anatomy workers exposed to FA, an increased frequency in both epithelial cells was found in exposed subjects. More recently in a group of Portuguese histopathology professionals Ladeira *et al*. (2011) reported elevated MN frequencies in PBLs and buccal epithelial cells, confirming previous reports by Costa *et al*. (2008, 2011) and Viegas *et al*. (2010). Ladeira *et al*. (2011) also evaluated the frequency of BNbud, finding a significant increase in FA- exposed workers compared to controls. BNbud formation in cells is associated to the elimination of amplified DNA and/or DNA-repair complexes (Fenech *et al*., 2011). Indeed, a recent study showed a significant association between a polymorphic gene in repair pathway and nuclear buds formation (Ladeira *et al*., 2013). Elevated frequencies of MN were also found among FA-exposed workers from industrial units both in PBLs (Jiang *et al*., 2010) and in nasal mucosa cells (Ballarin *et al*. 1992; Ye *et al*., 2005).

In the present work the correlation between MN frequencies in buccal cells and PBLs showed to be highly significant ($r=0.359$, $p<0.001$). The concomitant increase of MN formation in buccal cells (a FA-target tissue) and in PBLs indicates not only that inhaled FA is able to induce cytogenetic alterations in circulating systemic cells (distal tissue) but also that the damage between this two tissues may be associated, reinforcing the biological plausibility of inhaled FA to induce systemic genotoxic effects.

Finally, in our study, a positive significant correlation was observed between MNL frequencies and FA exposure duration ($r=0.277$, $p=0.011$). Also, by correlation testing a significant positive association was found between FA-level and MNB ($r=0.407$, $p=0.001$). Moreover, MNL and BNbud frequencies were increased with increasing FA-level of

exposure classes (Table XIV), confirming MN as a sensible bioindicator for the evaluation of the genotoxic action of FA.

SCEs are known to be increased by exposure to various genotoxic carcinogens (Albertini *et al*., 2000). Our results on SCE frequency (Table VI) agrees with data from other studies which reported a higher frequency of this cytogenetic biomarker in FA exposed workers compared to referents. Yager *et al*. (1986) were the first to observe an increase of SCE in subjects exposed to FA (mean level, 1.2 ppm) after a 10 week anatomy class compared to samples obtained from the same individuals immediately before FA exposure began. Shaham *et al*. (1997, 2002) reported similar findings in two studies involving pathology workers exposed to FA, both groups of workers showed significantly higher SCE frequencies compared to controls. In the larger study that enrolled 90 professionals, workers were divided into a “low exposure group” (mean level, 0.40 ppm) and a “high-exposure group” (mean level, 2.24 ppm); no significant differences on SCE frequencies were found between the two FA-exposed groups. More recently, Ye *et al*. (2005) examined two different types of workers exposed to FA, with different outcomes. One group was composed of 18 workers of a FA manufacturing facility (8h-TWA level, 0.82 ppm) whereas the second group included 16 waiters who were exposed to low levels of FA while working in a new ballroom for 12 weeks (5h TWA level, 0.09 ppm). The first group showed a significantly increased frequency of SCE in lymphocytes when compared to controls, whereas no significant difference was found in waiters, probably due to the low level of exposure to FA. Nevertheless, other studies also did not find increased SCE rates in workers exposed to FA (Thomsom *et al*., 1984; Suruda *et al*., 1993; Ying *et al*., 1999). Despite some negative findings, most of the studies have reported positive results, confirming this biomarker sensitivity to evaluate occupational exposure to FA (IARC, 2012).

The increase of SCE in PBLs of FA-exposed individuals may indicate the inability of the cell to efficiently repair replication-blocking lesions induced by the exposure, which results in fork collapse and the initiation of homologous recombination (HR) for repair. Moreover, there is some evidence that implicates the presence of single-strand breaks (SSBs) in DNA as the primary lesion that leads to SCE formation. The mechanism is likely to involved an unrepaired SSB, a DSB formation upon replication fork collapse, and HR (with the sister chromatid) to facilitate accurate repair (Wilson and Thompson, 2007). An increase of repair activity leads to an increase of SCE, which may explain the elevated frequency of this biomarker in PBLs of FA-exposed workers. Interestingly, we found significant positive associations, although weak, between this endpoint and double-strand

breakage parameters, namely Total-CAs (r=0.172; p<0.05), CTAs (r=0.159; p<0.05) and gaps (r=0.156; p<0.05). In conclusion, the increase on SCEs frequency found in the present study and previous reports may reflect the repair of FA- induced DNA lesions by HR.

Compared to the cytogenetic endpoints above described, the comet assay was recently introduced as a useful technique in human biomonitoring studies, allowing the evaluation of DNA damage (single and double strand breaks and abasic sites) at the single cell level. Hence, there are only a few studies published on FA occupational exposure in which this biomarker is used. In our study, the levels of DNA damage measured as percentage of tail DNA (%TDNA) were significantly increased in PBLs of FA-exposed professionals compared to controls (Table VI). This result agrees with Yu *et al*. (2005) and Jiang *et al*. (2010) who reported a significantly increase in comet assay parameters, comet tail length and olive tail moment, in workers exposed to FA (mean level, 0. 83 ppm) from two plywood factories. In contrast, no significant differences in comet assay endpoints was found in PBLs of workers from fiberboard plants compared to controls, however the results obtained in this study may be attributed to the low level of exposure to FA, 0.2 ppm (Aydin *et al*., 2013).

Comet assay has also proved to be a sensitive biological indicator in the evaluation of the genotoxic effect of FA in several *in vitro* experiments using cell lines or cells from FA-exposed rodent or humans (Frenzilli *et al*., 2000; Emri *et al*., 2004; Im *et al*., 2006; Liu *et al*., 2006; Sul *et al*., 2007). Interestingly, in most of these studies FA showed a “two-phase” dose-response relationship. At low doses, FA induced an increase in DNA migration whereas at higher doses a decrease in DNA migration was observed.

The DNA damaging and crosslinking effects of FA may explain this finding. Hence, at low concentrations, it induces strand breaks whilst at higher concentrations crosslinking activity seems to become the dominant lesion (Frenzilli *et al*., 2000). This is further explained by excess of FA-induced lesions at high concentrations which may overwhelm the cell repair capacity and result in the accumulation of cross-link lesions and the decrease in DNA migration; while the strand breaks observed at low concentrations are indicative of repair processes in progress or incomplete. Indeed, SSBs are often intermediates during repair of other DNA lesions. In fact, *Saccharomyces cerevisiae* strains defective in excision-repair of pyrimidine dimers showed to be more susceptible to the lethal effects of FA and a reduced capacity to undergo single-strand breaks compared to the wild type, which seems to suggest that SSBs are a step in the repair process of FA-induced lesions (Magaña-Schwencke *et al*., 1978; IARC, 2006).

In the present study, no significant differences were found regarding TCR-Mf (Table VI). To our knowledge, this is the first study in which TCR-Mf was evaluated in workers exposed to FA. Although this assay has shown to be a sensitive indicator for exposure to radiation and chemicals in some studies (Kubota *et al*., 2000; García-Lestón *et al*., 2012) others did not find the same result (Chen *et al.*, 2006).

4.4.2 **Immune-markers: Lymphocytes subpopulations**

The percentages of the different lymphocyte subsets in peripheral blood of exposed and control subjects were assessed as indicators of possible FA exposure-related immunotoxicity. The reference ranges for lymphocyte subsets in Caucasian population are approximately, 60-87% for total T lymphocytes $(CD3)^+$, 32-61% for T-helper cells $(CD4^+)$, 14-43% for T-cytotoxic cells $(\%CD8^+)$, 5-20% for B cells $(\%CD19^+)$ and 4-28% for NK cells $(CD16^+\text{-}56^+)$ (Santagostino *et al*., 1999). Our results in both groups fall within these expected ranges and are also in accordance with recent data published for a Portuguese population by García-Lestón *et al*. (2011).

Regarding the FA exposure effect when comparing both groups, no statistically significant differences were observed for total T lymphocyte and T-helper cells (Table XI). However, in the exposed group a significant increase in the percentages of T-cytotoxic cells and NK cells were found along with a significant decrease of percentage of B cells (Table XI). A lower ratio of $CD4^+/CD8^+$ (Th/Tc) was also observed with statistical significance, due to increase of T-cytotoxic cell subpopulation in the exposed group.

Our findings are in agreement with other studies, which showed that FA exposure may affect immunological parameters. Tang *et al*. (2009) summarised eight studies concerning FA-induced immunotoxicity and hematotoxicity. In one of the studies regarding clinical pathology personnel (FA exposure-levels between 0.2 and 0.8 ppm) a significantly higher proportion of the exposed subjects (14%) showed abnormal white blood cell (WBC) counts compared to controls (5%). Moreover, an investigation by Zhang *et al*. (2010b) on the ability of FA to disrupt haematopoiesis revealed a significant decrease in total WBC counts, granulocytes and lymphocytes in a group of FA-exposed workers (mean FA exposure-level, 1.28 ppm). In a recent study involving fibreboard plant workers exposed to low-levels of FA (mean level, 0.2 ppm) no significant differences in peripheral blood cells in terms of total WBC, red blood cells, neutrophils, and monocytes were found; nevertheless significant alterations in total lymphocyte and lymphocyte subpopulations were detect between workers and controls (Aydin *et al*., 2013)**.** Conversely, in other studies lymphocyte subsets and WBC counts were not significantly influenced by FA

exposure (Tang *et al*., 2009). It should be noted, however, that the available data on immunotoxicity and hematotoxicity in FA-exposed populations are still sparse, which may contribute to the inconsistent data.

In our study, the increased percentages of T-cytotoxic cells and NK cells may indicate an activation of the immune response in workers exposed to FA at a mean level of 0.38 ppm.

T-cytotoxic lymphocytes are cells enrolled in cell-mediated immune responses. Their function is to destroy compromised or stressed cells (e.g. infected cells or tumour cells) by recognition on the cell surface of a specific antigen presented by MHC class I (present in all nucleated cells). NK cells are effector lymphocytes of the innate immune system that have also cytotoxic function but do not form immune memory. NK cells are able to detect compromised cells tagged by antibodies or deficient in MHC class I surface marker. Recent research highlights the fact that NK cells are also regulatory cells engaged in reciprocal interactions with dendritic cells, macrophages, T cells and endothelial cells (Viver *et al*., 2008). Upon activation, T-cytotoxic lymphocytes and NK cells migrate to the site of inflammation or injury where they may exert their cytolytic function depending on the combination of inhibitory and activating signals conveyed by the target.

In fact, mild nasal epithelium lesions and a decrease in nasal epithelial cells have been consistently reported in workers exposed to FA (Edling *et al*., 1988; IARC, 2006). Moreover, FA is a mucous membrane irritant as demonstrated in experimental and human studies (IARC, 2006). A non-specific, non-allergic pro-inflammatory effect of inhaled FA was suggested (Pazdrak *et al*., 1993), and clinical findings of upper respiratory tract inflammation were reported in workers exposed to FA (Lyapina *et al*., 2004). Substances that elicit inflammatory events may provide a ‘danger’ signal to the immune system leading to activation of lymphocytes and other cells involved in immune response. Therefore, it is possible to speculate that the observed increase of T-cytotoxic lymphocytes and NK cells in exposed subjects may occur in response to the irritant properties of FA in the upper respiratory tract mucosa and tissue inflammation. Similarly, a significant increase on percentages of total T lymphocytes and NK cells were recently reported in workers exposed to 0.2 ppm, confirming our findings (Aydin *et al*., 2013).

Our data also show a significant decrease of %B cells in the exposed individuals compared to controls. In workers exposed to benzene and polycyclic aromatic hydrocarbons, B cells revealed to be more sensitive than T cells to the genotoxic effects of these compounds (Sul *et al*., 2002, 2003), which may probably be the case of FA and would explain our findings.

Further, the observed decrease in B cells suggests immunosuppression and decreased ability to respond to exogenous aggressions (e.g. virus, bacteria) in FA-exposed individuals. B cells are involved in organism's humoral response against antigens and engaged in the adaptive immune response system that enables a quick response of the organism to a relapsed infection. Upon activation, B cells differentiate into plasma cells producing specific molecules, antibodies, which eliminate targeted antigens (Janeway *et al*., 2001).

Although FA-exposed subjects showed as a whole increased %NK cells with regard to the controls, when analysing the effect of work-related factors on the endpoints studied, we found a clear decrease in these cells associated with the highest FA-levels of exposure (Table XIV), specifically at levels equal or above 0.35 ppm, showing percentages lower than the control population, which may indicate that at high exposure levels FA induce a decrease in immune response.

Indeed, a decrease in NK cells was reported for workers exposed to mean-airborne concentrations of 1.28 ppm (Hosgood *et al*., 2012). In a recent study, FA exposure in mice (10 ppm for 6h/day and 5 days/week) caused a selective NK cell deficiency, without any defect in other lymphocytes, by impairing NK cell differentiation (Kim *et al.*, 2013).

NK cells function comprises the control of several types of tumours and microbial infections by limiting their spread and subsequent tissue damage (Vivier *et al*., 2008).

Our findings suggest that workers exposed to FA are potentially more susceptible to infectious agents. Indeed, several studies describe a reduced resistance to infections (upper respiratory tract infections, recurrent rhinitis and pneumonitis) in individuals occupationally exposed to FA (IARC, 2006). In the study of Zhang *et al*. (2010b), 40% of the exposed subjects had recent respiratory infections. Lyapina *et al*. (2004) measured neutrophil respiratory burst activity (NRBA) in 29 workers exposed to FA (mean level, 0.71 ppm). Exposed workers showed a significant increase in upper respiratory tract inflammations compared to controls, but no significant differences in spontaneous or stimulated NRBA assays were found between them.

Further, viral associated cancers are increased in immunosuppressed individuals, most likely due to the inability of the organism to limit viral replication and/or expansion of infected cells (Schulz, 2009; Hosgood *et al*., 2012). The Epstein-Barr virus (EBV) is strongly implicated in the aetiology of nasopharyngeal carcinoma (Gullo *et al*., 2008). In fact, the risk of nasopharyngeal carcinoma in workers exposed to FA was more pronounced among EBV seropositive individuals than non-seropositive (Hildesheim *et al*., 2001).

Although our results are in agreement with published data, lymphocyte subpopulations were differently affected in few other studies. For example, in a group of 23 medical students exposed to FA (mean FA-level of exposure, 0.41ppm) during an 8-week anatomy class, Ying *et al.* (1999) found a significant increase in B cells and a significant decrease in total T cells, T-helper-inducer cells and T-cytotoxic-suppressor cells. Similarly, Ye *et al*. (2005) reported a significant increase in B cells and decrease in total T cells and T-cytotoxic-suppressor cells in 18 workers from a FA manufacturing facility (mean FA-level of exposure, 0.98 ppm). Interestingly in both studies, the pattern of immune alteration was the same at lower (0.41 ppm) and higher (0.98 ppm) levels of FA exposure. The small sample size or/and the FA level of exposure may have contributed to the different outcomes observed between these studies and the present work. Another factor that may also contribute to explain the different results is individual susceptibility. One of the enzymes involved in FA detoxification is the mitochondrial aldehyde dehydrogenase-2 (ALDH2) (Teng *et al*., 2001). *ALDH2* gene has an inactive *ALDH2*2* allele; the presence of the mutant allele leads to the decrease or absence of ALDH2 catalytic activity (Wang *et al*., 2002; Brennam *et al*., 2004). Approximately 50% of East Asians carry the mutant inactive *ALDH2*2* allele (Brennam *et al*., 2004; Oota *et al*., 2004), whereas nearly all Caucasians carry the functional *ALDH2*1/1* genotype (Chambers *et al*., 2002; Brennam *et al*., 2004). Furthermore, in a recent Chinese study FA-exposed workers carrying the wild-type genotype had a significant increment in formic acid in urine compared with workers carrying the mutant genotype, confirming that *ALDH2* genotypes may influence FA metabolism (Cheng *et al*., 2008b). Therefore, genetic variables may modulate the response to FA exposure and affect differently the populations exposed.

Overall, our findings indicate that inhaled FA may induce alterations in lymphocyte subsets in exposed workers.

These results should be carefully interpreted until confirmation in further studies. Although study population was homogenous regarding major confounding factors, and the statistical analysis took them into account, it is known that the lymphocyte subpopulations can vary depending on different life situations (Moszczynski *et al*. 2001).

Also of note, modifications in the immune parameters do not always give measurable effects. Moreover, even if an immunotoxic effect is shown, it does not always imply clinical consequences (Vial *et al*., 1996).

4.4.3 **Effect of lifestyle factors and parameter-specific confounders on the biomarkers studied**

In human biomonitoring studies is important to assess the influence of major potential confounding factors such as gender, age and smoking habits in the biomarkers studied.

4.4.3.1 ***Biomarkers of genotoxicity***

In the present study, age was found to be a confounder for CSAs, MNL and MNB, with frequencies increasing significantly with aging (Tables VIII and IX). This influence was further confirmed by the significant positive correlation found between age and MNL and MNB frequency. Our data is in accordance with previous studies (Carbonell *et al*., 1996; Bolognesi *et al*., 1997; Bonassi *et al*., 2001, 2011) reporting a positive association between age and cytogenetic biomarkers. The observed age-related effect is probably associated with a progressive increase in spontaneous chromosome instability and the loss of efficiency in DNA repair mechanisms, which may result in the accumulation of genetic lesions with increasing age (Bolognesi *et al*., 1999).

Cigarette smoking had a significant influence on aneuploidy in PBLs (decrease) and MNB frequencies in oral mucosa (increase) (Table IX). Further, the significant correlations found between these biomarkers and packs/year confirmed the observed effect of tobacco smoking on these endpoints.

Tobacco smoke contains a high number of mutagenic and carcinogenic substances, such as benzene, arsenic and FA. Epidemiologically it has been associated with a higher risk for cancer development, especially in the oral cavity, larynx, and lungs, as these are places of direct contact with the carcinogenic tobacco compounds (IARC, 2004).

With regard to our data, the significant decrease of aneuploid PBLs found in smokers might be related with a higher apoptotic activity in these subjects induced by the increased levels of DNA damage resulting from tobacco smoking. Indeed, exposure of rats to mainstream cigarette smoke produced a significant and time-dependent increase in the proportion of apoptotic cells in the bronchial and bronchiolar epithelium (D'Agostini *et al*., 2000). Some authors have reported lower damage in PBLs of healthy smokers compared to never-smokers (Wang *et al*., 2003; Lao *et al*., 2008). Furthermore, smokers have showed an increase on baseline repair capacity (Wei *et al*., 2000a), probably as an adaptation resulting from the increased demand for repair stimulated by the continuous damage caused by tobacco carcinogens. The genotoxicity of mainstream tobacco smoke and cigarette smoke condensate has been demonstrated *in vitro* and *in vivo* experiments, although human studies have produced mixed results (DeMarini, 2004). Nevertheless, it is

important to note that the unbalanced number of smokers and non-smokers in our study limits the value of the data obtained and restricts possible conclusions, so larger studies are necessary to confirm this result.

Several studies have described an increase on MN formation in exfoliated buccal cells of smokers (Goud *et al*., 2004; Gabriel *et al*., 2006; Proia *et al*., 2006; Nersesyan *et al*., 2011). In fact, smoking was the only lifestyle variable that significantly influenced the level of micronuclei in the oral mucosa of 120 healthy individuals, with smokers showing higher frequency of MNB compared to controls (Konopacka *et al*., 2003). Furthermore, our results showed a significant positive association with packs/year. Similarly, among a group of tobacco smokers Wu *et al*. (2004) found a significant positive trend between MNB frequency and either daily cigarette consumption or cumulative smoking pack-years. Yet other publications report no difference between smokers and non-smokers (Karahalil *et al*., 1999; Nersesyan *et al*., 2006; Holland *et al*., 2008). Nevertheless, a recent meta-analysis found significant increases in the MNB frequency but only in heavy smokers (40 cigarettes/day) (Bonassi *et al*., 2011).

In our study, fruit consumption was found to significantly decrease the DNA damage measured by comet assay %TDNA parameter (Table X). Epidemiological studies consistently report that a balanced diet rich in fruit and vegetables is associated with a reduced risk of cancer and heart disease (Duthie *et al*., 2006). Indeed, fruits are rich in several phytochemicals and antioxidants such as vitamin C and flavonoids that inactivate reactive oxygen species (ROS) involved in the initiation or progression of these chronic diseases (Brevik *et al*., 2011a). Therefore regular fruit consumption protects against the oxidative damage of DNA and thus might prevent mutation and cancer. Human supplementation trials investigating the anti-oxidant effect of fruit consumption measured by comet assay (classical and modified versions) have, however, given ambiguous results (Hoelzl *et al*., 2009). In some studies, kiwifruit (juice or whole fruit) consumption showed to significantly decrease the levels of DNA breakage in lymphocytes (induced by H_2O_2) and formation of endogenous oxidised bases (measured by incubation with lesion-specific enzymes) indicating an increased antioxidant capacity with fruit intake (Collins *et al*., 2001; Collins *et al*., 2003; Brevik *et al*., 2011b). Conversely, in other trials, no effect in oxidative damage or repair in PBLs was observed after fruit consumption (Duthie *et al*., 2006; Yuan *et al*., 2011). Our data suggest that regular consumption of fruit may actually protect against DNA damage, however, one shortcoming of our study was the lack of information concerning the type of fruit consumed which would enable further conclusions.

4.4.3.2 ***Lymphocyte subpopulations***

In the present study significant influence of the major confounding factors, gender and age, were observed for some lymphocyte subpopulations (Table XII).

Previous studies have demonstrated gender-related differences in immune cell percentages (Santagostino *et al*., 1999; Jentsch-Ullrich *et al*., 2005; Yan *et al*., 2010). The total lymphocyte count is generally similar in males and females, but the percentage of T cells within the lymphocyte population tends to be lower in males (Yan *et al*., 2010) mostly because of total T lymphocyte and T-helper cells decreases (García-Dabrio *et al*., 2012). Percentage of T-cytotoxic cells usually does not differ between males and females (Jentsch-Ullrich *et al*., 2005). However, in our study males had a significantly lower percentage of T-cytotoxic cells compared to females. No significant differences were found between genders for T lymphocytes and T-helper cells. Some evidence suggests that the influence of gender on T lymphocyte subsets is related to sex hormones, although the processes involved are not well understood. Two different mechanisms have been proposed: acceleration of thymocyte apoptosis by androgens, which may mediate processes of thymocyte selection and shape the peripheral T cell repertoire (Olsen *et al*., 1998) or the effect of binding to cell receptors for the sex steroids present on T cells (Chng *et al*., 2004, Pierdominic *et al*., 2010).

The influence of age in the immune system has been described in several studies (Jentsch-Ullrich *et al*., 2005; Saule *et al*., 2006; Andreu-Ballestar *et al*., 2012). Age is known to affect the immune function and the percentages of circulating lymphocytes in a process often designated as "immunosenescence" (Yan *et al*., 2010). Aging of the immune system is characterised by a decrease in immunocompetence. Hence, it may lead to infections or autoimmune diseases and be involved in the pathogenesis of several age-related disorders, such as cardiovascular or neurodegenerative disorders (Laffon *et al*., 2013). In our study, a significant decrease with age was found for T-cytotoxic cells whereas total T lymphocytes and T-helper cells were significantly increased (Table XII). The effect of age in these endpoints was further confirmed by correlation analysis. Other authors (Tollerud *et al*., 1990; Chng *et al*., 2004) found similar results. Moreover, in a recent study carried out in a Mediterranean population (Spain) a significant positive correlation with age was found for T-helper cells, in addition, an inverse trend was observed for T-cytotoxic cells, but it did not reach statistical significance (García-Dabrio *et al*., 2012). The impact of age on lymphocyte subsets is not well established with different studies reporting contrasting results (Bisset *et al*., 2004; Coelho *et al*., 2011; García-Lestón *et al*., 2011; Andreu-Ballester *et al*., 2012). T lymphocyte generation in aging is known to result from dynamic changes in thymic as well as extrathymic functions, along

with sequential developmental steps from stem cells to ultimately mature cells (Jentsch-Ullrich *et al*., 2005). Therefore, the age range of subjects studied, methodological features, environmental and genetic factors may influence the results and account for the differences (Jentsch-Ullrich *et al*., 2005; Andreu-Ballester *et al*., 2012).

In our study, multivitamin supplement intake was found to significantly increase T-cytotoxic cells percentage (Table XII). Furthermore, fruit consumption was also found to increase T-helper cells percentage, but it did not reach significance. The link between diet and immunity has long been established. Systematic studies have consistently shown that nutritional deficiency impairs immune response, particularly cell-mediated immunity by reducing number and functions of T-cells, phagocyte function, cytokine production, secretory antibody response, antibody affinity and complement system (Chandra, 1999). Similar findings have been reported for moderate deficiencies of individual nutrients such as trace minerals and vitamins, particularly Zn, Fe, Se, vitamins A, B_6, C and E (Chandra, 1999; Liu *et al*., 2011). The use of nutrient supplements, singly or in combination, stimulates immune response and may lower the incidence of infection or reverse some of the changes associated with impaired immune responses in some chronic diseases (Grzegorzewska and Leander, 2005) and in elderly people (High, 2001). In a randomised placebo-controlled intervention study, healthy volunteers consumed probiotic multivitamin and mineral supplement for 3-5 months during winter/spring period. Leukocytes, lymphocytes, in particular T-lymphocytes including T-cytotoxic cells and T-helper cells, as well as monocytes were significantly increased in the “supplementation” group, during the first two weeks of supplementation compared to placebo. Additionally, in the “supplementation” group the incidence and severity of symptoms related to common cold, frequent at that time of year, were reduced. Overall, according to published data, multivitamin supplementation intake and a balanced diet (rich in vitamins and anti-oxidants) improve the immune status, which is in agreement with our findings.

4.4.3 **Correlations between biomarkers studied**

The association found between MNL frequencies with CAs parameters and gaps is related to the fact that both endpoints are able to indicate chromosomal and chromatidic breaks, which suggests chromosome breakage as the primary mechanism of FA-related micronuclei formation. In fact, in an earlier study by Titenko-Holland *et al*. (1996) it was detected a greater increase in centromere-negative micronuclei content in buccal and nasal tissues of FA-exposed students, however Orsière *et al*. (2006) found higher frequencies of centromere-positive micronuclei (monocentric) in health professionals

exposed to FA. Our findings, comply with recent studies by Speit *et al*. (2011) (mammalian cell lines) and Costa *et al*. (2011) (FA-exposed pathology anatomy workers) indicating a clastogenic action of FA, thus confirming previous results from Titenko-Holland *et al*. (1996). A significant association was found between CSAs and %TDNA, but the coefficient was relatively low. This association is not surprising since %TDNA detects DNA strand breaks, including double-strand breaks.

Concerning the immune markers, T-cytotoxic lymphocytes were inversely correlated with B cells ($r=-0.577$, $p<0.001$). As mentioned earlier, B lymphocytes seem to be more sensitive to genotoxicity induced by organic compounds exposure than T lymphocytes (Sul *et al*., 2002, 2003). The primary function of T-cytotoxic lymphocytes is to remove compromised or stressed cells. The inverse correlation found may result as a response to stressed B cells. Further, T-cytotoxic lymphocytes were positively associated with genotoxicity endpoints, while B cells were inversely correlated. Genotoxicity events have been shown to alter immune responses (Gasser and Raulet, 2006). No previous studies were found reporting similar results. Nevertheless, our findings indicate that increased levels of DNA damage are associated with alterations in immune system.

5. BIOMARKERS OF SUSCEPTIBILITY

5.1 OVERVIEW

5.1.1. Genetic polymorphisms as susceptibility biomarkers

About 99.9% of the DNA is identical in every human genome (Hirvonen *et al*., 2008). The 0.1% difference is responsible for the inter-individual variation and the unique phenotype of each individual. These minor differences correspond to genetic variations in the DNA sequence, known as polymorphisms. The majority of polymorphisms are single nucleotide polymorphisms (SNPs), changes at a single DNA base, which occur with a frequency of 10^{-6} throughout the human genome (Rueff *et al*., 2002). SNPs play an important role in modulating susceptibility to diseases as well as in the individual response to various drugs and environmental carcinogens (Hemminki and Shields, 2002).

In simple terms, SNP's can alter an individual's response to a chemical exposure by interfering with the expression or function of proteins involved in their metabolism or in the repair of the chemical-induced DNA lesions. SNPs in gene coding regions can lead to changes in the biological properties of the encoded protein. In fact, about 50% of the SNPs in the coding regions results in a change in the amino acid protein sequence (Hirvonen *et al*., 2008). On the other hand, SNPs in non-coding regulatory regions may affect gene expression levels in an allele-specific manner (Wang *et al*., 2005).

Altered function of low penetrance genes due to SNPs may affect the gene-environment and gene-gene interaction, thereby increasing the risk of cancer development (Hirvonen *et al*., 2008). Therefore, the identification of sequence polymorphisms that modulate gene expression or function is crucial for understanding human genetic differences and diseases (Pastinen *et al*., 2004) and to recognise susceptible groups in the population.

Genetic polymorphisms are thus considered to play a primary role in individual response to carcinogen-induced disease (Teixeira *et al*., 2004). Therefore, in recent years, they have been used as susceptibility biomarkers in human biomonitoring studies to assess the risk of exposure to genotoxic chemicals.

5.1.2. Genetic Polymorphisms, DNA damage and Cancer Risk

Cancer is a multifactorial disease that results from complex interactions between genetic and environmental factors (Hirvonen, 2008). This means that with few exceptions (e.g. Mendelian cancer syndromes such as retinoblastoma) the cause is not entirely genetic or

entirely environmental (Rueff *et al*., 2002) and is not a single gene or single environmental factor that has large effects on cancer susceptibility (Kiyohara *et al*., 2007). Instead, various genetic and biologic aspects (e.g. gender, ethnicity) and exogenous factors (e.g. environmental/occupational exposure to carcinogenic compounds, lifestyle factors, nutritional status) play a joint role in the process of carcinogenesis (Rueff *et al*., 2002) (Figure 16).

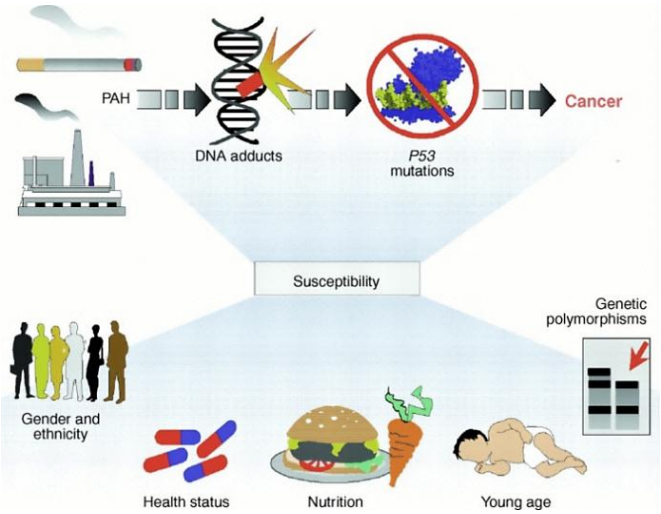


Figure 16. Both environmental and host factors acts in concert in individual susceptibility (genetic and acquired) for disease development (*retrieved from Perera, 1997)*

Biologically, cancer is considered to be a genetic disease, caused by genetic alterations at either chromosomal or gene levels, as identified in most cancers (Wei *et al*., 2000b). It is also agreed, that genetic polymorphisms are associated with most common disorders with a genetic component (Rueff *et al*., 2002). Nevertheless, as mentioned before, susceptibility-conferring alleles are not sufficient to cause disease, but modulate the risk in combination with other alleles and environmental exposures.

It is known that environmental carcinogens, as a result of complex metabolism, are able to interact with DNA and produce measurable alterations evaluated by several biomarkers of lesion (e.g. DNA adducts, chromosomal aberrations, micronucleus) (Rueff *et al*., 2002; Mateuca *et al*., 2008). Interestingly, it has also been observed that the correlation between the levels of the different biomarkers and levels of exposure are not always linear, suggesting that intra-individual susceptibility play an important role in processing the damage (Rueff *et al*., 2002). Indeed, several studies have shown that the level of biomarkers arising from environmental and/or occupational exposure may be influenced by polymorphic genes coding for enzymes engaged in metabolic or repair processes (Pavanello and Clonfero, 2000; Hung *et al*., 2005; Mateuca *et al*., 2008). Therefore, the

complex metabolism of carcinogens, involving different polymorphic genes, as well as gene variants of DNA repair mechanisms, can modulate the individual susceptibility for cancer (Pavanello and Clonfero, 2000). This is best exemplified by tobacco smoking; cigarette smoking is one main cause of lung cancer but only a relative minority of smokers develop pulmonary cancers.

5.1.3. **Xenobiotic-metabolising enzymes**

The balance between phase I and phase II metabolic enzymes is an important determinant of whether exposure to carcinogens will result in toxicity or increased levels of genetic damage (Talalay *et al*., 1990).

Most carcinogens undergo activation by phase I enzymes, often as an oxidation reaction, and detoxification by phase II enzymes. The cytochrome P450 enzyme superfamily constitutes the majority of phase I enzymes, whilst the glutathione S-transferases (GSTs) and N-acetyltransferases (NATs) are primarily responsible for detoxification of xenobiotics in phase II.

Phase I enzymes are generally involved in the metabolism of xenobiotic compounds with hydrophobic character, producing more polar intermediates that are easily excreted from the cell. However, phase I metabolism may also result in the activation of carcinogenic compounds, yielding reactive electrophilic metabolites that can react with cellular macromolecules, like DNA. The action of phase II enzymes, generally contributes to detoxifying metabolism of more hydrophilic carcinogens. Phase II enzymes, also act on phase I carcinogenic metabolites, transforming them into inactivated compounds that are then excreted. Less frequently, GSTs and NATs can also mediate activating steps for some carcinogen metabolites.

Genetic polymorphisms exist in a number of phase I (activating) and phase II (inactivating) enzymes. Hence, it is plausible that individuals with genotypes associated with a more efficient activating enzyme and/or a less efficient inactivating enzyme might be at particularly high risk of adverse health effects, if exposed to toxicants. Indeed, accumulated data suggest that polymorphisms in genes controlling carcinogen metabolism and thus the metabolic capacity may in fact play a primary role in susceptibility to environmentally induced diseases, such as cancer.

5.1.3.1. ***Polymorphisms of phase I enzymes studied: Cytochrome P450 family***

The Cytochrome P450 (CYPs) system is the most important enzymatic system involved in phase I of human metabolism (Rueff *et al*., 2002). CYPs constitute a superfamily of a

highly diverse group of haem-containing enzymes responsible for the oxidative metabolism of a wide range of drugs and toxic hydrocarbons. All CYPs exhibit similarity in their structure and general mechanism of action; NADPH is required as a coenzyme and O_2 is used as a substrate. However, there are significant differences in substrate specificity due to structural variations at the active site. Based on sequence homologies, human CYPs are divided into families and subfamilies.

The highest concentration of CYPs is in the liver, where it determines the intensity and duration of drug action and promotes the detoxication of some xenobiotics. Extrahepatic tissues, especially those that are portals of entry for foreign compounds, such as the respiratory tract, also express xenobiotic-metabolising CYPs. In these tissues, CYPs not only contribute to the first-pass clearance but may also influence the tissue burden of foreign compounds or bioavailability of therapeutic agents. In the cell, these enzymes are primarily located in the membrane of the endoplasmatic reticulum (microsomes), but they are also found in the mitochondrial membrane.

During biotransformation, xenobiotics are either detoxified or activated into reactive intermediate substances that might be more toxic than the parent compounds. However, not all xenobiotic-metabolising CYPs subfamilies show the same propensity in the bioactivation of chemicals. For example, the CYP2B, 2C and 2D subfamilies play virtually no role in the bioactivation of toxic and carcinogenic chemicals, whereas the CYP1A, 1B and 2E subfamilies are responsible for the bioactivation of the majority of xenobiotics (Ioannides C, 2007).

These reactions can also result in the transfer of electrons to produce oxygen radicals. Cytochrome P450-catalysed reactions can thus produce xenobiotic metabolites and oxygen species capable of interfering with cell homeostasis. Over 60 key forms of CYPs are known, with hundreds of genetic variations that correspond to different metabolic activities and therefore to a wide variety of susceptibility to potential deleterious effects induced by toxicants (Autrup H, 2000; Godschalk and Schooten, 2008).

CYP2E1 gene (CYP2E subfamily)

The CYP2E subfamily has only be described in mammals, and only one CYP2E gene, *CYP2E1* has been identified in humans (Godschalk and Schooten, 2008).

CYP2E1 gene encodes the enzyme N,N-dimethylnitrosamino-N-dimethylase, which catalyses the metabolism of a wide variety of therapeutic agents, toxicants and low molecular weight pro-carcinogens, including benzene, N-nitrosamines and vinyl chloride (Anzenbacher *et al*., 2001). It has a clear toxicological role, since it may activate pro-

carcinogens, organic solvents and drugs, and potentiate their toxicity by converting them into cytotoxic or carcinogenic products (Nedelcheva *et al*., 1996).

For instance, this enzyme is involved in the oxidation of compounds, such as ethanol, producing reactive oxygen species (ROS), which can affect target tissue and ultimately lead to carcinogenesis (Guo *et al*., 2008). Indeed, a number of studies in cells lines have established the importance of this enzyme in oxidative stress (Cederbaum, 2006). During ethanol oxidation, the CYP2E1 enzyme produces ROS ($O2^{-}$ and H_2O_2) that can deplete glutathione and cause cellular damage (Gonzalez, 2007). In addition, ROS can further react and produce oxidants or result in elevated lipid peroxides that can form adducts with cellular nucleophiles such as proteins and nucleic acids (Gonzalez, 2007).

The enzyme is mostly expressed in the hepatic tissue and can be induced by xenobiotics such as ethanol, acetone or isoniazid (Novak and Woodcroft, 2000) all of which are substrates. It was also detected, at low levels in human lung and oesophagus (Ding and Kaminsky, 2003), and evidence suggests that it can be present in kidney and nasal mucosa (Lucas *et al*., 2001).

CYP2E1 enzyme was detected by immunochemistry in the white cell fraction of human peripheral blood (Lucas *et al*., 2001). Expression of the CYP2E1 enzyme in lymphocytes appears to be influenced by the same factors that affect the concentration of the hepatic enzyme, including xenobiotics and physiological status (Raucy *et al*., 1995; 1997).

The *CYP2E1* gene is present in the human population in various polymorphic forms. Although there are a number of SNPs in the *CYP2E1* sequence, no known variant results in the complete inactivation of the gene, suggesting that the enzyme is essential for humans (Gonzalez, 2007).The gene is located in the 10q24.3-qter region of the chromosome 10 (Shahriary *et al*., 2012). It spans 11,413 base pairs and contains 9 exons, 8 introns, and a typical TATA box (Shahriary *et al*., 2012). The first alleles were identified by restriction fragment length polymorphism (RFPL) analysis. The best studied *CYP2E1* polymorphisms were detected by *Pst*I/*Rsa*I and *Dra*I restriction enzymes, which corresponds to SNPs in the 5'-flanking region and in the intron 6 of the *CYP2E1* gene. It has been shown that polymorphisms detectable by *Pst*I/*Rsa*I can affect the transcription of *CYP2E1 in vitro*. In contrast, *Dra*I polymorphism does not appear to affect gene transcription, but rather the CYP2E1 enzyme catalytic activity (Shahriary et al., 2012).

The allele frequencies of *CYP2E1* polymorphisms may differ remarkably among different human populations (Garte *et al.*, 2001; Bolt *et al*., 2003). For instance, the *DraI* polymorphism occurs at a higher frequency in Taiwanese when compared with European and African-Americans (Stephens *et al*., 1994). Additional information on genotype

frequency distribution of the polymorphism studied is shown in Annex III. Polymorphisms in the *CYP2E1* gene have been associated with increased risk of cancer in different sites, including breast cancer (Anderson *et al*., 2012; *CYP2E1* intron, allele A), lung cancer (Wu *et al*., 1997; *RsaI*, allele), nasopharyngeal carcinoma (Kongruttanachok *et al*., 2001; *RsaI*, allele C) and gastric cancer (Boccia *et al.*, 2007; *PsI/RsaI*, allele C).

5.1.3.2 ***Polymorphisms of Phase II enzymes studied: Glutathione S-transferases***

The glutathione S-transferases (GSTs) play a critical role in providing protection against electrophiles and products of oxidative stress (Hayes and Pulford, 1995).

The GSTs are a superfamily of phase II enzymes which catalyse the conjugation of reduced glutathione (GSH) with different species of electrophilic compounds, including many environmental mutagens and carcinogens and their reactive metabolites (Strange *et al*., 2001). In addition to their role in phase II detoxification, GSTs also play an important role in modulating the induction of other enzymes and proteins for cellular functions, such as DNA repair (Hayes and Pulford, 1995). Because of their role in the detoxification of potentially carcinogenic compounds, these enzymes and the genes encoding them may play an important role in cancer susceptibility.

GST enzymes are present in the cytosol of all eukaryotic cells, but are also bound to membranes (Landi, 2000). The human cytosolic GSTs are grouped into eight families of GST isoenzymes, including: GSTA (α), GSTM (μ), GSTP (π), GSTS (σ), and GSTT (θ) (Raimondi *et al*., 2006; Hirvonen, 2008). The classification is in accordance with the substrate specificity, chemical affinity, structure, aminoacid sequence and kinetic behavior of the enzyme (Hayes and Pulford, 1995). Because GSTs have overlapping substrate specificities, the deficiencies of a given GST isoenzyme may be compensated by other isoenzyme and use of alternative metabolic pathways is possible (Hirvonen, 2008). Polymorphisms have been identified in the *GSTM1*, *GSTT1* and *GSTP1* genes coding for GSTs enzymes in the mu (μ), theta (θ), and pi (π) classes, respectively. These enzymes detoxify a wide variety of chemicals, such as reactive epoxides and carcinogens produced by tobacco smoke. Genetic polymorphisms in GSTs encoding genes have been associated with susceptibility to genetic lesions in populations exposed to several environmental carcinogens, including polycyclic aromatic hydrocarbons (PAHs; Butkiewicz *et al*., 2000), styrene (Teixeira *et al*., 2004) and hydroquinone (Silva *et al*., 2004).

Many studies have suggested a predisposing role of *GSTs* polymorphisms for various cancers in different tissues, such as lung, breast and colon (Hirvonen, 2008). However, there are also various reports contradicting these findings (Raimondi *et al*., 2006). One of

the reasons of the controversial data may be the overlapping substrate specificities of the GSTs enzymes. Therefore, simultaneous determination of different *GSTs* genotypes relevant for a given exposure may be crucial for a reliable interpretation of the role of the *GST* gene family in cancer development (Hirvonen, 2008). Indeed, meta-analyses studies have indicated that carriers of both *GSTM1* and *GSTT1* null alleles have a slightly higher risk of developing lung cancer compared to carriers of at least one functional allele (Benhamou *et al*., 2002; Raimondi *et al*., 2006).

Additional information on frequency distribution of the *GSTs* studied among different racial groups is available in Annex III.

Glutathione S-transferase T1 gene (GSTT1)

The encoded GSTT1 enzyme (θ subfamily) is involved in the detoxification of monohalomethanes and reactive epoxide metabolites of butadiene, both of which are constituents of tobacco smoke (Landi *et al*., 2000; Hirvonen, 2008).

GSTT1 gene is located on 22q11.23 region of the chromosome 22; it spans 8092 base pairs and contains 5 exons and 4 introns (Yang *et al*., 2013). The most common polymorphism in *GSTT1* consists of a deletion of the whole gene (*GSTT1*0*, null genotype) resulting in the lack of active enzyme (Raimondi *et al*., 2006). The risk associated with the null genotype is difficult to predict since the enzyme may have both detoxification and toxification activities toward different industrial and environmental chemicals. The enzyme in human erythrocytes detoxifies the carcinogen ethylene oxide but may enhance the formation of the genotoxic metabolites from dichloromethane (To-Figueiras *et al*., 1997).

The prevalence of *GSTT1* null genotype shows a wide variation among different racial populations and ethnic groups. For instance, in Caucasians the prevalence is 10–20% (Hirvonen, 2008) and in Asians it can vary between 45-60% (Cho *et al*., 2005).

The null genotype has been correlated with increased risk of several types of malignancies including lung and larynx cancers (Yang *et al*., 2013, Sørensen *et al*., 2004; Russo *et al*., 2013). A modest increase in the risk of haematological malignancies (lymphoma, leukaemia) was also observed (Ye and Song, 2005; Bin and Luo, 2013). However, other studies have reported negative results (López-Cima *et al*., 2012; Sørensen *et al*., 2007). Some of the observed heterogeneity may be partly due to ethnicity (Raimondi *et al*., 2006).

Glutathione S-transferase M1 gene (GSTM1)

A gene cluster at 1p13.3 region of chromosome 1 encodes the GST μ subfamily (Hirvonen, 2008). One of the gene variants includes a deletion and homozygotes (GSTM1*0, null genotype) do not express the protein, thus lacking the enzyme activity (Hayes and Pulford *et al*., 1995). GSTM1 enzyme is involved in the detoxification of large hydrophobic electrophiles, such as benzo(a)pyrene and other PAHs found in tobacco smoke.

Several epidemiological studies on *GSTM1* null genotype have suggested, although moderate, an association between this genotype and cancer risk, namely bladder cancer, lung cancer, prostate cancer and head and neck cancer (Hirvonen, 2008). Moreover, this genotype appears to be associated with a modest increase in the risk of acute lymphoblastic leukaemia (Ye and Song, 2005). Some conflicting reports also exist (López-Cima *et al*., 2012; Bin and Luo, 2013). However, recent pooled and meta-analyses support previous findings suggesting that GSTM1 deficiency can contribute substantially to the incidence of cancer at the population level (Engel *et al*., 2002; Hiyama *et al*., 2008; Mo *et al*., 2009).

The distribution of the *GSTM1* null genotype varies among racial and ethnic groups. For instance the mean reported frequency for the null genotype among Caucasians is about 50%, while for African descendants is 29 % (Hirvonen, 2008).

Glutathione S-transferase P1 gene (GSTP1)

The glutathione *S*-transferase P1 (GSTP1) belongs to GSTP (π) subfamily. A single 2.8 kb long *GSTP1* gene at 11q13 region of chromosome 11, encodes an isoform that is known to metabolise many carcinogenic compounds, including polycyclic aromatic hydrocarbons (PAHs). GSTP1 is the most abundant glutathione S-transferase in the lungs (Cote *et al*., 2009) and therefore it is thought to be of particular importance in the detoxification of inhaled carcinogens (Hirvonen, 2008).

An A>G transition at nucleotide 313 of the *GSTP1* gene results in an isoleucine (Ile) to valine (Val) amino acid change at codon 105 (Ile105Val). This produces a variant enzyme, which differs in enzymatic activity (more or less effective detoxification) depending on the substrate and alters their thermo stability (Mo *et al*, 2009). The presence of the *GSTP1* Val variant (GG or GA genotypes) is associated with a lower conjugating activity when compared to the homozygous *GSTP1* Ile variant (AA genotype) (Cote *et al*., 2009). Moreover, having at least one copy of the G allele at this locus is also associated with

increased levels of hydrophobic adducts in the lung and higher levels of PAH-DNA adducts in human lymphocytes (Butkiewicz *et al*., 2000).

The *GSTP1* Ile105Val polymorphism has been investigated for associations with breast, head and neck cancers, with both positive and negative outcomes (Egan *et al*., 2004; Ruwali *et al*., 2011). In addition, epidemiological studies of the impact of the *GSTP1* Ile105Val polymorphism on lung cancer risk including two meta-analyses show inconsistent results (Cote *et al*., 2009; López-Cima *et al*., 2012).

5.1.4. **DNA Repair**

DNA repair is a ubiquitous process throughout the living world and is essential for the maintenance of genome integrity. Several identified genes and sequence variants are involved in human DNA repair (Au *et al*., 2003; Rueff *et al*., 2002). These genes function in a complex diverse set of pathways that involve recognition and removal of DNA lesions caused by endogenous or exogenous agents and/or correction of DNA errors occurred during DNA replication or DNA repair (Ronem and Glickman, 2001). Other genes are indirectly involved in DNA repair, by regulating the cell cycle, providing an opportunity for repair or controlling apoptosis mechanisms (Moses *et al*., 2001; Ronem and Glickman, 2001).

Both endogenous and exogenous agents are continually inducing cellular DNA damage. If unrepaired, the damage can interfere with important cellular functions potentially leading to genetic instability, unregulated cell growth and cancer (Au *et al*., 2003). On the contrary, if damage is recognised by cell machinery it can be repaired enabling the cell to replicate as planned. At cell level, several responses may occur to prevent replication in the presence of genetic errors: checkpoints can be activated to arrest the cell cycle, transcription can be up-regulated to compensate for the damage or cell can trigger apoptosis (Goode *et al*., 2002).

Polymorphisms in DNA repair genes may be innocuous or may change protein function and therefore alter individual's capacity to repair DNA lesions caused by exogenous or endogenous agents (Au *et al*., 2003). A deficient or unrepaired damage can ultimately lead to cancer (Ronem and Glickman, 2001).

Mutations that affect the function of DNA repair enzymes are rare in the human population because they cause severe health consequences including higher proneness for cancer development (Au *et al*., 2003). Examples of cancer syndromes where the disease-causing mutations occur in DNA repair genes include *xeroderma pigmentosum*, Fanconi anaemia

and Bloom's syndrome; each disorder shows specific damage sensitivities at the cellular level and specific disease phenotypes (Moses *et al*., 2001).

The etiologic association between deficient DNA repair and increased cancer risk is also supported by evidence from epidemiologic studies of sporadic cancers of the lung (Goode et al., 2002), head and neck (Gresner et al., 2012), bladder (Stern et al., 2002) and skin (Mocellin et al., 2009). These data strongly suggest that it is important for normal tissues to have efficient DNA repair in order to eradicate DNA lesions caused by carcinogens, thereby reducing the risk of mutation fixation and subsequent development of cancer (Wei et al., 2000b).

5.1.4.1 ***DNA Repair pathways***

A variety of complex pathways have evolved to perform the critical repair functions. The main classic pathways of DNA repair are base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and double-strand break repair (Jeppesen *et al*., 2011). Each pathway operates on specific types of DNA lesion and involves numerous molecules in the repair processes (Goode *et al*., 2002) (Figure 17).

BER (Figure 17A) operates on small lesions (e.g. oxidised or reduced bases; fragmented or nonbulky adducts) caused by a variety of factors including ionising radiation and alkylating agents (Goode *et al*., 2002). The process which aims to remove and replace a damaged nucleotide, is typically initiated by one of several substrate-selective DNA glycosylases, which recognise and excise base modifications, such 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), among others (Wilson *et al*., 2011). The resulting abasic site is incised by an AP endonuclease (APE1). The baseless phosphodiester backbone and adjacent nucleotides are then removed and the gap is filled by DNA polymerase β that uses the other strand as a template for the DNA synthesis (Collins, 1996). The final step is ligation of repair patch into the pre-existing DNA strand by a DNA ligase, which in mammals is typically ligase III in complex with x-ray cross-complementing protein 1 (XRCC1) (Almeida and Sobol, 2007).

NER (Figure 17B) is the major pathway in humans for the repair of bulky lesions, such as UV-induced pyrimidine dimers and chemical adducts, induced by environmental carcinogens (Au *et al*., 2003). In the NER repair process, the distortion of the double helix (e.g. bulky adduct) is recognised by a complex of bound proteins including XPC; the formation of the transcription factor complex, TFIIH, allows the unwinding of the DNA and the removal of the damaged single-stranded fragment (usually about 27–30 nucleotides);

the DNA synthesis is carried out by DNA polymerase δ using the other strand as a template (Goode *et al*., 2002).

The MMR (Figure 17C) maintains genomic stability by correcting replication errors, such as mispairings (base-base or insertion-deletion mismatched) caused by DNA polymerase during replication (Aquilina and Bignami, 2001; Goode *et al*., 2001). The process begins with specific repair proteins recognising and binding to the mismatched bases, further enzymatic action proceeds to exonucleolytic degradation, DNA resynthesis and ligation (Aquilina and Bignami, 2001).

Double-strand breaks, DSBs, (Figure 17D) can be produced by replication errors and by exogenous agents (e.g. ionising radiation) (Goode *et al*., 2002). DSBs are the most serious form of DNA damage because they pose problems for transcription, replication, and chromosome segregation. DSBs affect both strands of the DNA duplex and therefore prevent the use of the complementary strand as a template for repair. Cells have evolved at least two different mechanisms of DSBs repair: Homologous recombination (HR) and Non-homologous end-joining (NHEJ). The first is a highly accurate repair process that corrects DSBs defects in an error-free manner using a mechanism that retrieves genetic information from the homologous, undamaged DNA molecule. The majority of HR-based repair takes place in late S- and G2-phases of the cell cycle when an undamaged sister chromatid is available for use as repair template (Khanna and Jackson, 2001). Conversely, the NHEJ repair mechanism is error prone, since it re-joins the two broken ends of DNA directly, which frequently can lead to small alterations (base pair substitutions, insertions and deletions) in the DNA sequence (Khanna and Jackson, 2001).

The Fanconi Anaemia (FANC) pathway has emerged recently as an important model for studying the mechanisms surrounding DNA damage response/DNA repair, genomic instability and ubiquitin signalling (Moldovan and D'Andrea, 2009; Kottemann and Smogorzewska, 2013).

FANC repair pathway coordinates the action of multiple distinct repair processes, in particular, NER, HR and translesion synthesis (TLS; a mode of damage tolerance that uses specialised polymerases to insert a base across from a lesion or abasic site) (Moldovan and D'Andrea, 2009). Studying the FANC repair pathway provides a unique window into the elaborate interplay of multiple cellular networks involved in DNA repair. The FANC proteins work together to maintain genomic stability during DNA replication and to repair DNA interstrand crosslinks (ICLs), a severe lesion that blocks both DNA

replication and transcription (Moldovan and D'Andrea, 2009; Kottemann and Smogorzewska, 2013).

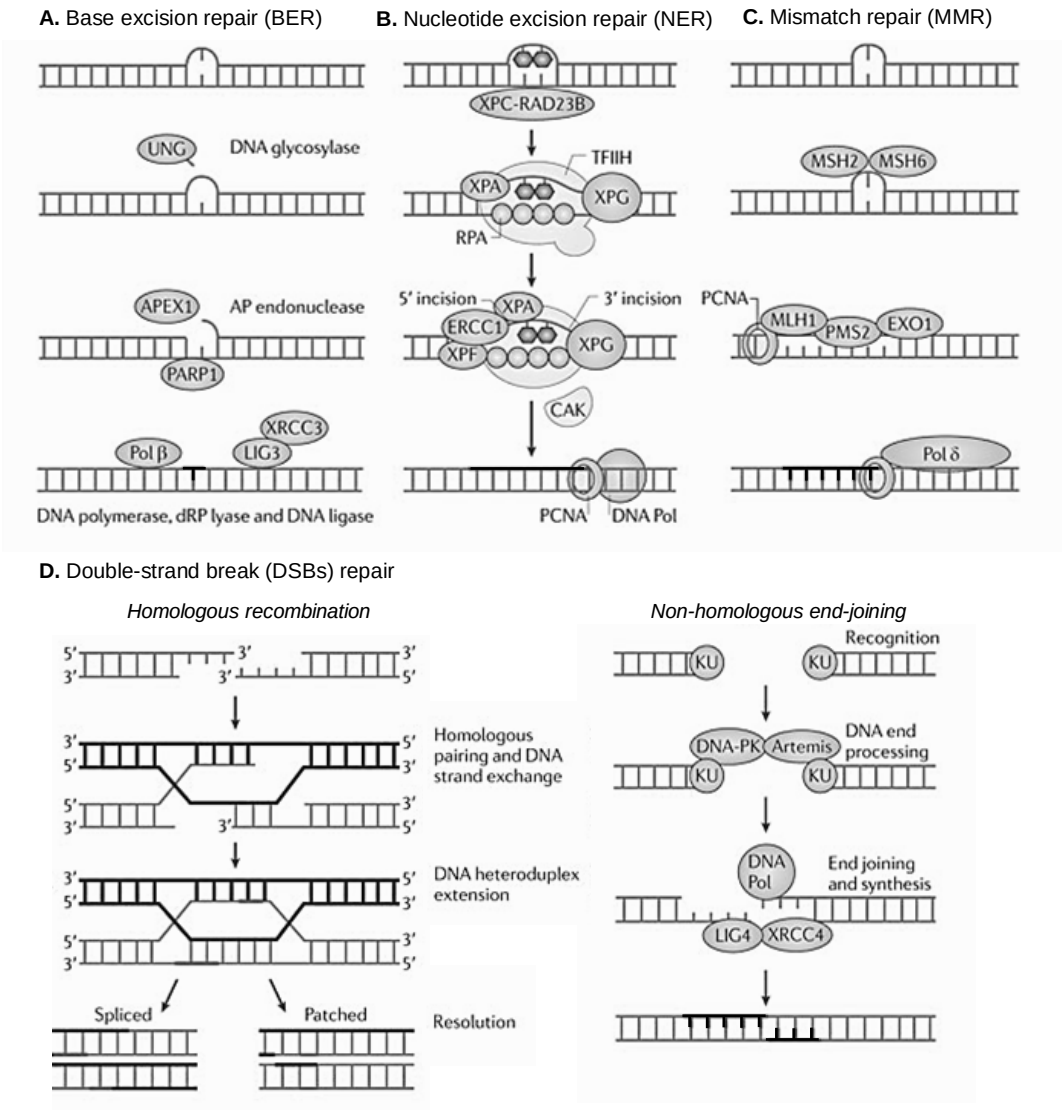


Figure 17. DNA Repair pathways. **A)** BER mediates the removal and replacement of small lesions, such as, damaged bases and uracil residues caused by reactive oxygen species (UNG for uracil is shown in the figure); the resulting abasic site is incised by an AP endonuclease (APEX1); the baseless phosphodiester backbone is removed and the gap filled by DNA polymerase β (Pol β). **B)** NER acts on larger lesions or adducts and involves lesion recognition, formation of the TFIIH complex, removal of 25–30 nucleotides and gap filling by a DNA polymerase. **C)** MMR removes mismatched bases; after recognition of specific proteins, a segment of DNA is excised between the mismatch and a nearby nick, the gap left is filled by DNA polymerase δ. **D)** DSBs can be repaired by Non-homologous end joining (NHEJ), a error prone process that joins directly the DNA ends or Homologous recombination (HR), a error free repair processes that operates when a double-stranded copy of the sequence is available (*adapted from Lange et al., 2011)*

FANC is a rare genetic disease caused by germline mutations that disrupt the FANC pathway leading to a deficient repair function (Moldovan and D'Andrea, 2009).

FANC patients manifest a high propensity for cancer, namely squamous cell carcinomas (e.g. head and neck, gynecologic system) and acute myeloide leukaemia (AML) (Alan and D'Andrea A, 2010). This phenotype has launched the question as to whether inherited or acquired mutations in FANC genes might be involved in the development of sporadic cancers (Tischkowitz *et al*., 2004). Moreover, several genes involved in the FANC pathway also predispose individuals to breast and ovarian cancer, which, together with the cancer susceptibility of patients with FANC, suggests FANC proteins have an important role in suppressing tumorigenesis (Kottemann and Smogorzewska, 2013).

The association between a chromosomal instability syndrome and cancer predisposition is not surprising since excessive chromosome breakage might be expected to lead to unrepaired DNA damage and apoptosis, or to mutations which could confer a selective growth advantage to certain cells (Mathew, 2006). However, the reason why cells of myeloid origin are particularly affected in FANC case leading to AML is not known (Mathew, 2006; Moldovan and D'Andrea, 2009). Recent work has begun to gain tantalising insight into not only FANC pathogenesis, but also the intersection between DNA repair and stem-cell maintenance and the general role of FANC repair pathway genes (Kottemann and Smogorzewska, 2013). One explanation is that the myeloid precursors are particularly susceptible to forms of DNA damage (e.g. crosslinks), that are recognised or repaired by the FANC pathway. It is likely that the cellular endogenous metabolism might specifically yield agents that cause DNA lesions (Moldovan and D'Andrea, 2009), and circulating metabolites such as formaldehyde (FA) may account for the sensitivity of FANC hematopoietic stem cells (Ridpath *et al*., 2007). Indeed, recent advances have identified endogenous aldehydes, such as acetaldehyde and FA, as possible inducers of the DNA damage that may lead to the chromosomal instability seen in FANC pathway deficient cells (Rosado *et al*., 2011; Kottemann and Smogorzewska, 2013). More importantly, there is also recent evidence that genes in the FANC repair pathway have a crucial role in counteracting acetaldehyde-induced genotoxicity in mice, furthermore, mice mutant in both *FANC* repair genes and aldehyde-catabolism gene spontaneously developed acute leukaemia (Langevin *et al*., 2011).

Taking into account the above, it is reasonable to speculate the existence of polymorphic *FANC* genes that may modulate individual susceptibility to the effects of exposure to FA, particularly in the workplace. This association would also agree with Zhang *et al*. (2010) hypothesis of FA ability to affect haemotopoietic system in exposed individuals and help to elucidate the increase of AML cases among FA-exposed workers. Therefore, it was relevant in our study a screening of polymorphic genes associated with FANC repair pathway.

At least fifteen FANC complementation groups of proteins/genes (FANC-A, -B, -C, -D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L -M, -N/PALB2, -P and -O/RAD51C) were found to constitute the FANC pathway (Kim and D'Andrea, 2012).

The FANC pathway is turned on during the S phase of the cell cycle or following DNA damage, by FANC protein monoubiquination (the addition of one ubiquitin, an amino acid tag, to the internal lysine residue of a protein). During activation, multiple FANC proteins undergo phosphorylation by checkpoint kinases (ATR-CHK1) (Kim and D'Andrea, 2012). Once active, the FANC proteins work in concert to regulate the monoubiquitinated state of the FANCD2 and FANCI proteins and the downstream functions of the repair process (Alan and D'Andrea, 2010).

A few models on the FANC-mediated crosslink mechanism have been proposed, but generally it is described as follows. During replication, the presence of an ICL that covalently links both strands of a DNA duplex, blocks the DNA unwinding and stops the progression of the replication fork. A complex formed by FANCM and FAAP24 proteins recognises the stalled replication fork structure and recruits the FANC core complex to the damaged DNA region initiating the FANC pathway (Kim and D'Andrea, 2012) (Figure 18).

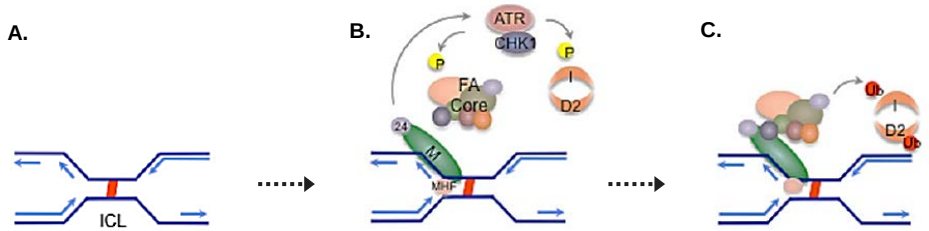


Figure 18. Interaction of the FANC proteins in the recognition of an ICL in S phase. **A)** Two replication forks converge on the DNA. **B)** The FANCM–FAAP24 complex recognises the stalled replication fork structure. FANCM stabilises the stalled replication fork, and initiates the ATR-CHK1-dependent checkpoint response, which in turn phosphorylates multiple FANC proteins, including proteins in the FANC core complex and FANCD2/FANCI unit. **C)** The FANC core complex monoubiquitinates FANCD2/FANCI complex, and it is recruited to the DNA lesion (*adapted from Kim and D'Andrea, 2012)*

FANCM prevents the collapse of replication fork, independently of FANC pathway activation. The FANC core complex is an ubiquitin ligase multi-subunit composed by eight FANC proteins (FANCA,-B,-C,-E,-F,-G,-L,-M) that monoubiquitinates the FANCD2/FANCI unit (Alan and D'Andrea A, 2010). Lastly, the ubiquitin-tagged FANCD2/FANCI complex interacts with other downstream FANC proteins (FANCD1, FANCN, and BRIP1) and other DNA repair proteins to resolve the ICL and the stalled replication fork (Kim and D'Andrea, 2012).

In the DNA, two nucleolytic incisions flanking the ICL region release it from one of the DNA strands. The unhooking process forms a double-strand break and leaves cross-linked oligonucleotides in the complementary strand. The latter is bypassed by TLS restoring the strand and NER removes the remaining adduct and fills the gap. The double-strand break is repaired by HR mechanism. An ubiquitin-specific protease removes the ubiquitin from the FANCD2/FANCI complex, and completes the FANC repair pathway (Kim and D'Andrea, 2012) (Figure 19).

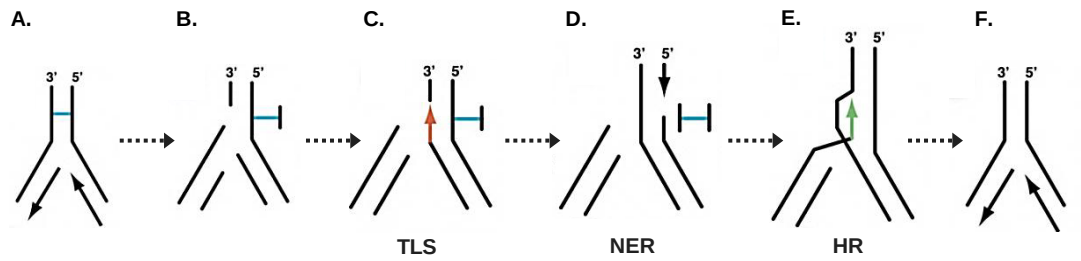


Figure 19. Simplified model of the mechanism of ICL repair process involving NER, TLS and HR. **A)** An ICL blocks the DNA unwinding and stops the progression of the replication fork; FANC complex stabilises the staled fork. **B)** The crosslink unhooking from one of the DNA strands forms a DSB and leaves a monoadduct in the complementary strand. **C)** FANC recruits TLS to bypass the remaining monoadduct. **D)** NER excises the monoadduct and repairs the strand. **E)** The DSB is repaired by HR mechanism. **F)** Intermediate DNA crossover structures are resolved and the replication fork is re-established *(adapted from Kennedy and D'Andrea, 2005)*

5.1.4.2. ***Polymorphisms of the BER pathway studied***

BER (base excision repair) repairs DNA lesions such as non-bulky base modifications, abasic sites and a range of chemically distinct single-strand breaks (Wilson *et al*., 2011). Genetic polymorphisms in BER pathway have been associated with cancer risk at several sites (Wilson *et al*., 2011; Hung *et al*., 2005). Further, deficiencies in BER gene products have been linked to neurodegenerative disorders and immunodeficiency (Doseth *et al*., 2011; Wilson *et al*., 2011). Because polymorphisms of BER genes may affect the level of DNA repair induced by chemicals, leading to carcinogenesis, it is important to establish the molecular causes of the carcinogenetic process. The major genes involved in the BER pathway include, among others, *XRCC1*, *PARP1* and *MUTYH* (Almeida and Sobol *et al*., 2007). Genotype frequencies may vary markedly across race, supplementary information on the frequency distribution of the studied polymorphisms is available in Annex III.

XRCC1

The XRCC1 (x-ray repair cross-complementing group 1) protein forms complexes with DNA polymerase β, DNA ligase III and poly-ADP-ribose-polymerase (PARP) in the repair

of nicks or gaps left in BER pathway and in the repair of single-strand breaks, SSBs (Aka *et al*., 2008). It is an important protein for co-ordination of DNA damage repair (Thaker and Zdzienickab, 2003).

The *XRCC1* gene has been located at 19q13.2–13.3 region of human chromosome 19. The importance of this gene is highlighted by the description of its null mutant mice, whose embryonic development is arrested (Aka *et al*., 2008). Cell lines defective in this nonenzymatic scaffold protein show reduced SSBs repair, increased sensitivity to DNA-damaging agents that produce SSBs (Kulkarni *et al*., 2008), and elevated chromosomal aberrations and sister chromatid exchanges frequencies (Thompson and West *et al*., 2000).

Two common polymorphisms of *XRCC1* that lead to amino acid substitutions are located in exon 10 (G to A, arginine to glutamine, Arg399Gln) and in exon 6 (C to T, arginine to tryptophane, Arg194Trp).

In humans, while the *XRCC1* Arg399Gln substitution is associated with increased genetic instability the *XRCC1* Arg194Trp substitution is suggested to enhance individual DNA repair capability (Tuimala *et al*., 2004; Aka *et al*., 2008; Chang *et al*., 2009).

Indeed, published data on *XRCC1* gene and cancer risk show associations between: a) the *XRCC1* 399Gln/Gln genotype and increased risk of tobacco-related cancers among light smokers, but decreased risk among heavy smokers (Hung *et al*., 2005); b) the *XRCC1* Arg194Trp substitution and reduced risk of various types of cancer, including breast and lung cancer (Goode *et al*., 2002). Nonetheless, recent data from a Polish study suggest that *XRCC1* 399Gln allele is a potential risk factor for breast cancer (Romanowicz *et al*., 2010). Yet, there are other studies with contradictory findings (Hirvonem *et al*., 2008). The *XRCC1* Arg194Trp substitution is more prevalent among Asians (2.6-13.2%) than among Caucasians (≤ 2.3 %) (Hung *et al*., 2005). Regarding *XRCC1* Arg399Gln genotype frequencies, there is no major variation across race and ethnicity (Hung *et al*., 2005; Chang *et al*., 2009).

PARP1

Human Poly(ADP-ribose) polymerase-1 (PARP1) is a major member of the PARP family proteins. PARP1 functions as a DNA damage sensor in the DNA repair process. Generally, in response to DNA damage caused by exogenous or endogenous agents, PARP1 binds to the DNA lesion through its DNA-binding domain. This activates the poly(ADP-ribosyl)ation of target proteins involved in the DNA repair pathways, including BER and DSBs systems, and recruits the repair proteins to the sites of DNA damage (Yu

et al., 2012). PARP1 complex interacts preferentially with XRCC1, triggering the BER repair machinery (Masutani *et al*., 2003).

PARP1 is also involved in other cellular processes, such as modulation of gene transcription, cell proliferation and death. Accumulated data from *in vitro* and *in vivo* studies indicates that PARP1 deficiency leads to DNA repair defects, genomic instability and failure to induce cell apoptosis, which may contribute to cancer development (Yu *et al*., 2012).

The human *PARP1* gene is located on 1q41-42; it spans 47.3 kb and comprises 23 exons. *PARP1* Val762Ala is a polymorphism in exon 17 (T to C transition) that may result in altered activity of PARP1, due to an amino acid substitution, alanine (Ala) for valine (Val), in the protein catalytic domain. *PARP1* Val762Ala has been implicated in human carcinogenesis, but with inconclusive results. Nevertheless, data of a recent meta-analysis suggest that this polymorphism may modulate cancer risk differently depending on ethnicity and type of cancers. The study found an association between *PARP1* Val762Ala and increased risk of cancer among Asians but a decreased risk among Caucasians, particularly of glioma (Yu *et al*., 2012).

MUTYH

The *MUTYH* gene encodes a DNA glycosylase involved in the repair of oxidative DNA lesions such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), a stable oxidation product of guanine. If left unrepaired, 8-oxo-dG can mispair with adenine during DNA replication, leading to a G:C>T:A transversion (Santos *et al.*, 2012). The MUTYH protein prevents these transversions by excising any adenine residue misincorporated in the newly synthesized DNA strand opposite to 8-oxo-dG, thus allowing the repair process to be continued by downstream BER enzymes (Mazzei *et al.*, 2013). MUTYH dysfunction may, therefore, be especially problematic for tumorigenesis in humans since there are no other mechanisms for repairing 8-oxo-dG/adenine mismatches (Santos *et al.*, 2012). Mutations in this gene strongly predispose humans to a rare hereditary form of colorectal cancer (MAP) (Out *et al.*, 2012). MAP tumours display an unusually high proportion of somatic G:C>T:A transversion mutations in certain genes, probably reflecting defective MUTYH protein activity and consequent failure to correct A:8-oxo-dG mispair (Santos *et al.*, 2012). Several *MUTYH* germline mutations and SNPs have been identified (Wilson *et al*., 2001). *MUTYH* is located on the short arm of the chromosome 1 at 1p34. It spans a region of 11,147 base pairs with 16 exons.

MUTYH Gln335His (rs3219489) is a common polymorphism located on exon 12. The variant genotype implicates a G> C transition and a substitution of the amino acid glutamine (Gln) for histidine (His) at the 335 codon of the MUTYH protein. The enzyme encoded by this variant has been demonstrated to have partially impaired glycosylase activity *in vitro* (compared to the wild-type, the variant enzyme was 34% less active in removing adenine from substrates containing an A:GO mismatch) and could therefore contribute to cancer susceptibility (Santos *et al.*, 2012).

The 335His variant allele has been suggested to be associated with increased risk of colorectal cancer in some studies (Picelli *et al.*, 2010; Santos *et al.*, 2012). A positive association was also found for lung cancer, but only when gene-gene interactions were considered (Qian *et al.*, 2011). Also, in a bladder cancer susceptibility study, gene-gene interactions among BER polymorphisms (including *MUTYH* Gln335His) were observed in ever smokers (Huang *et al.,* 2007), suggesting that BER genetic variation might contribute to cancer risk through gene-gene and gene-environmental interactions (Santos *et al.*, 2012).

5.1.4.3. ***Polymorphisms of the DSBs repair pathway studied***

Unrepaired or misrepaired, double-strand breaks (DSBs) may result in cell death, chromosome rearrangements and genome instability, all processes involved in carcinogenesis. The DSBs repair by homologous recombination (HR) is a key pathway for the maintenance of genetic stability in mammalian cells (Tambini *et al*., 2010). HR is a high fidelity process that uses homologous sequence as a template to synthesise new error-free DNA. It acts in coordination with the S and G2 checkpoint machinery to eliminate chromosomal breaks before the cell division occurs (Thompson and Schild, 2001). Two major cancer genes, BRCA1 and BRCA2, associated with breast cancer are involved in HR pathway. Gene variants of other participants in HR have showed to be associated with cancer risk at several sites, pointing out the relevance of HR and DSBs in cancer susceptibility. The frequency distribution of DSBs repair genotypes varies across racial groups, supplementary information for each polymorphic gene studied is available in Annex III.

Rad51

The RAD51 protein is responsible for the central activity of the HR repair pathway, in which it catalyses the invasion of the broken ends of the DSBs into the intact sister

chromatid, that will serve as template to reconstruct the missing part of the damaged strand (Gresner *et al*., 2012).

In this process, the generated single-stranded DNA ends are bound by Rad51 to form a nucleoprotein filament that searches the undamaged DNA on the sister chromatid for a homologous repair template. Other proteins including the breast cancer predisposition proteins (BRCA1 and BRCA2), and several additional Rad51-related proteins (e.g. XRCC2, XRCC3) serve as accessory factors in filament assembly and subsequent Rad51 activities (Richardson, 2005; Thacker, 2005). Furthermore, BRCA2 is shown to regulate both the intracellular localisation and the DNA-binding ability of this protein (Davies *et al*., 2001). Several studies of Rad51 among BRCA1/2 mutation carriers have found associations with breast cancer risk (Thacker, 2005; Ricks-Santi *et al*., 2011). In addition, several genetic assays have also demonstrated that p53 can down-regulate Rad51-mediated HR events; however, the process by which it occurs remains unclear (Richardson, 2005)

The role of Rad51 in genomic integrity is strongly supported by *Rad51* mouse knockouts which result in early embryonic lethality (Richardson, 2005). Experimental studies have also show that the loss of Rad51 may drive to genetic instability, chromosomal aberrations, and carcinogenesis by facilitating an accumulation of genetic changes (Richardson, 2005). A recent case control found evidence that *Rad51* gene variability may be of relevance with respect to head and neck cancer risk modulation (Gresner *et al*., 2012)

The *RAD51* gene is located at 15q15.1 in chromosome 15. Multiple transcript variants encoding different isoforms have been found for this gene (Thacker, 2005).

The *RAD51* SNP in the 5′ untranslated region (G > T, rs1801321, *RAD51* 5'UTR) is a polymorphism whose association with cancer risk is scarcely explored. The few available epidemiological data concerns breast cancer risk, but no association was found (Silva *et al*., 2010).

XRCC2

The X-ray repair cross-complementing protein group 2 (XRCC2) is a RAD51-related protein, essential for efficient HR repair process of DSBs (Tambini *et al*., 2010). The degree of sequence homology to RAD51 or to other RAD51-like proteins is relatively small, around 45% similarity and 25% identity (Thaker and Zdzienickab, 2003).

XRCC2 role is still not clear, but there are indications that it acts as a cofactor for the RAD51 in strand invasion and exchange activities and also in late stages of the HR repair

pathway (Richardson, 2005; Silva *et al*., 2010). The loss of *XRCC2* has showed to result in a severe delay in the early response of RAD51 to DNA damage (Tambini *et al*., 2010).

In addition, *XRCC2*-deficient cell lines have chromosome missegregation associated with fragmentation of the centrosome, indicating that the protein is also required for correct chromosome segregation (Griffin *et al*., 2000).

The disruption of *XRCC2* gene in mice resulted in early embryonic lethality (Thaker and Zdzienickab, 2003).

Human *XRCC2* gene was first identified by the ability to complement the irs1 mutated hamster cell line phenotype, which is highly sensitive to mitomycin C, a DNA cross-linking agent, and suffer increased frequency of chromosomal aberrations, i.e., breaks (Cui *et al*., 1999). Further studies showed that *XRCC2* is highly conserved, and most truncations of the protein destroy its ability to protect cells from the effects of the DNA cross-liking agents (Park *et al*., 2012).

XRCC2 gene is localised on chromosome 7q36.1. The *XRCC2* Arg188His polymorphism (G > A, substitution of arginine to histidine, rs3218536) has been linked to small but significant increased risk to breast (Thompson and Schild, 2002; Silva *et al*., 2010), colorectal (Vineis *et al*., 2009) and cervical cancer (Pérez *et al*., 2013). Other studies reported null results (Yu *et al*., 2010; Park *et al*., 2012). Therefore, additional studies with larger sample size are required to confirm the role of this polymorphism in cancer susceptibility.

XRCC3

The XRCC3 (x-ray repair cross-complementing group 3) protein plays a critical role in repair of DSBs in homologous recombination pathway. It is involved in the assembly and direct stabilisation of RAD51 protein multimers at DSBs sites (Aka *et al*., 2008, Tambini *et al*., 2010); however the precise function of XRCC3 is not fully understood (Richardson, 2005). Additionally, there is evidence that XRCC3 protein is required for correct chromosome segregation (Griffin *et al*., 2000). Analysis of sequence similarities showed that XRCC3 is a RAD51-like protein, but it only shares around 25% sequence identity to RAD51 and to other RAD51-related proteins (Thaker and Zdzienickab, 2003).

As in *XRCC2*, the human *XRCC3* gene was originally identified by the ability to correct the phenotype of mutated rodent cell lines (irs1SF) for sensitivity to ionising radiation and other DNA damaging agents (e.g. cisplatin, mitomycin C) (Thaker and Zdzienickab, 2003). Without XRCC3 activity, the irs1SF cells displayed highly elevated levels of deletions, breaks, and translocations (Cui *et al*., 1999).

The human *XRCC3* gene is located at 14q32.3 region of chromosome 14. Rare microsatellite polymorphism in the *XRCC3* have been reported, including a C>T non-conservative change (Thr241Met, rs861539) in exon 8, which gives rise to an amino acid substitution (threonine for methionine) (Aka *et al*., 2008). The *XRCC3* 241Met allele was associated with significant increases in chromosome deletions in x-ray-challenged blood lymphocytes (Manuguerra *et al*., 2006). Several studies have evaluated the influence of *XRCC3* Thr241Met on cancer risk in different tissues (Goode *et al*., 2002). Positive associations were obtained for bladder cancer (Matullo *et al*., 2001), breast cancer (Silva *et al*., 2010), lung cancer and colorectal cancer (Aka *et al*., 2008). In some studies, it was found a potential gene-gene interaction with genes of metabolic enzymes (Matullo *et al*., 2001) and other DNA repair pathways (Aka *et al*., 2008). Nevertheless, other studies have reported contrasting results (Manuguerra *et al*., 2006; Aka *et al*., 2008). The prevalence of Met/Met homozygosis is around 4.6 % in African Americans, 0.2 % in Asians, and 12.4 % in Caucasians (Manuguerra *et al*., 2006).

5.1.4.4 ***Polymorphisms of the FANC repair pathway studied***

FANC (Fanconi anaemia) is a genetic recessive disorder, caused by mutations in genes regulating replication-dependent removal of DNA crosslinks (Moldovan and D'Andrea, 2009).

At least fifteen FANC complementation groups (*FANC-A, -B, -C, -D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L -M, -N/PALB2, -P and -O/RAD51C*) and their corresponding genes have been identified to cooperate in the FANC pathway (Su and Huang, 2011).

The members of the FANC complementation group do not share sequence similarity. Four of the FANC pathway genes (FANCD1/BRCA2, -J/BRIP1, -N/PALB2, and -O/RAD51C) are known breast cancer susceptibility genes and other gene products, that connects FANC proteins to HR (homologous recombination) of DSBs repair pathway (Castella *et al*., 2011). Proteins encoded by these genes participate in the repair of ICLs during replication and are essential for accurate processing of stalled replication forks (Castella *et al*., 2011).

Mutations in FANC-A, -C and -G are the most common and account for approximately 85% of patients with FANC (Su and Huang *et al*., 2011). FANC individuals have greatly increased risks of cancer, including acute myeloid leukaemia (AML).

It has long been hypothesized that heterozygotes for autosomal recessive DNA repair disorders may have reduced efficiency of their DNA repair systems that could cause an increased risk of cancer (Tischkowitz *et al*., 2008). Epidemiological studies assessing

incidence of malignancies (including AML) in FANC families have failed to demonstrate an increased incidence in heterozygous individuals (Mathew, 2006; Tischkowitz *et al*., 2008). However, this raises the question as to whether inherited or acquired mutations in FANC genes might be involved in the development of sporadic cancers, e.g. AML (Tischkowitz *et al*., 2004). The major role of *BRCA2/FANCD1* in susceptibility to breast and other cancers, such as cancer of the ovarian , prostate and pancreas, is well established (Mathew, 2006). There have been several studies of the other known *FANC* genes in relation to breast cancer susceptibility, some of which found an association with *FANCN/PALB2*, *FANCJ/BRIP1* and *FANCD2* (Mathew, 2006; Barroso *et al*., 2009). There is also some limited data suggesting an association with sporadic hematological malignancies (Tischkowitz *et al*., 2004). It is important to point out that these studies had some limitations and therefore replication in larger studies is needed to confirm these findings (Mathew, 2006). Additional information on frequency distribution of the polymorphisms studied among different racial groups is available in Annex III.

FANCA

FANCA (or Fanconi anemia complementation group A) protein is one of the eight FANC proteins that comprise the FANC core complex, a multisubunit complex required for FANCD2 ubiquitination, essential for resolving ICL and stalled replication fork, during replication.

Furthermore, evidence suggests that FANCA phosphorylation is DNA damage–inducible and essential to the function of the FANC pathway (Collins *et al*., 2008).

Mutation in *FANCA* gene accounts for more than 60% of all cases of FANC (Moldovan and D'Andrea, 2009). The mutation profile of *FANCA* is highly heterogeneous with over 100 different mutations described, a large part being deletions (Castella *et al*., 2011). Hence is one of the most studied genes in the *FANC* family in relation to cancer proneness.

Human *FANCA* gene is located at 16q24.3 in chromosome 16. The *FANCA* Thr266Ala polymorphism (rs7190823) results in a aminoacid substitution, in codon 266, of threonine to alanine. The current evidence regarding the role of FANCA mutations in sporadic malignancies is limited. Nonetheless, some findings suggest that acquired FANCA dysfunction and/or *FANCA* variants may be involved in the promotion of genetic instability in cases of sporadic AML (Lensch *et al*., 2003; Tischkowitz *et al*., 2004). Additionally, a recent epidemiologic study have found an increased risk of cervical cancer associated with FANCA variants (Wang *et al*., 2009b).

BRIP1

BRIP1 (BRCA1-interacting protein 1) also known as BACH1 (BRCA1-associated C-terminal helicase-1) or FANCJ (for Fanconi anemia complementation group J) is a DNA helicase that interacts directly with BRCA1 and thus is essential for DNA repair and genomic stability (Cantor and Guillemette, 2011). It is universally expressed and co-localised with BRCA1 in the nuclear foci (Ma *et al*., 2013).

Phosphorylated BRIP1 functions together with BRCA1 protein to mediate proper and efficient repair of DNA double-strand break via HR repair subpathway (Cantor *et al*., 2001). BRIP1 interacts with BRCA1, unwinds DNA structures that block replication forks and facilitates checkpoint signalling (Constantinou, 2012). The complex formed by BRIP1 and BRCA1 is important not only for HR-mediated double-strand break repair but also for tumour suppressor function (Ma *et al*., 2013).

This specific interaction between BRIP1 and BRCA1 is regulated by the cell cycle and is essential for execution of the G2/M cell cycle checkpoint and for normal progression through S phase by assisting in the resolution of stalled replication forks (Kumaraswamy and Shiekhattar, 2007)

It is well established that a deficient DNA helicase function can result in human genetic disorders in which genomic instability and predisposition to cancer are common features (e.g. Bloom Syndrome) (Cantor *et al*., 2004). Accumulating data suggests an anti-oncogenic role of BRIP1, and downregulation of *BRIP1* gene has been reported in multiple cancers (Cantor *et al*., 2001; Ma *et al*., 2013).

The *BRIP1* gene is located on chromosome 17q22, it spans 180 kb and comprises 20 exons (Cantor *et al*., 2001). In the last few years, common SNPs in the *BRIP1* gene have been associated with susceptibility to breast cancer (Cantor *et al*., 2004), ovarian cancer (Pennington and Swisher, 2012) cervical cancer (Ma *et al*., 2013) and prostate cancer (Kote-Jarai *et al*., 2009).

Recently, the rare polymorphism *BRIP1* Ser919Pro (substitution of serine to proline, rs4986764) was reported to be a risk factor of breast cancer (Sigurdson *et al*., 2004) especially in women up to age 50, however contrasting results was also found (Vahteristo *et al*., 2006) and therefore this association needs further research.

A final model linking the various factors involved in individual's response to an exogenous insult and potential biologic effects is outlined in Figure 20.

In short, most of the carcinogens present in the environment undergo bio-activation into ultimately reactive metabolites, and this transformation is modulated by the individual

genetic profile of metabolic genes. In addition, common polymorphisms in DNA repair genes may alter protein function and eventually the individual ability to repair DNA damage (Iarmacovai *et al*., 2007).

Therefore, the study of the interaction of different genetic polymorphisms involved both in the metabolism of environmental carcinogens and in the DNA repair is crucial to evaluate the possible combined effect of several genetic variants in relation to a specific chemical exposure (Mateuca *et al*., 2008).

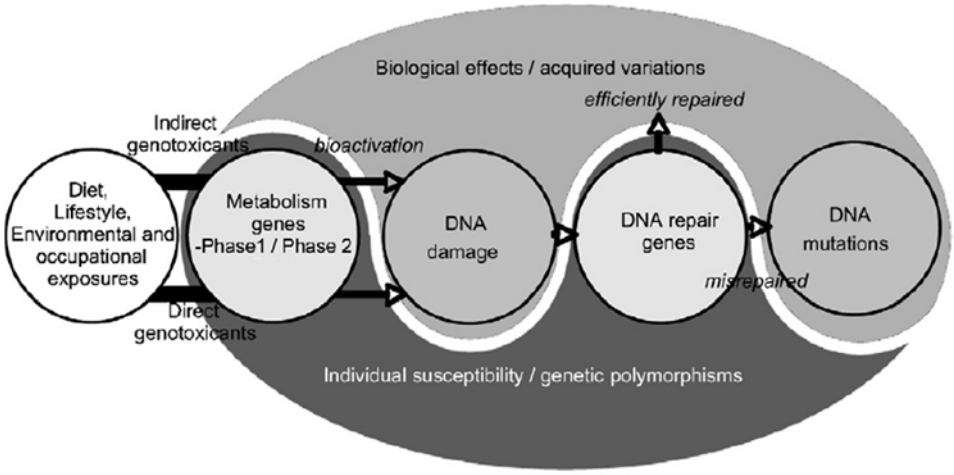


Figure 20. Association among the various factors involved in individual's response to an exogenous insult and potential biologic effects (*retrieved from Iarmacovai et al. (2007)*

5.2 MATERIAL AND METHODS

5.2.1 DNA extraction

Genomic DNA was obtained from heparinised whole blood samples (350 µL) using a commercially available kit (Qiagen EZ1 DNA Blood kit; Qiagen BioRobot EZ1 System) according to the manufacturer's instructions. DNA samples were stored at -20 ºC until analysis.

5.2.2 Genotyping of polymorphisms in gene involved in the metabolism

Detailed information regarding selected SNP's and corresponding amino acid exchanges are presented in Table XVI. Genotype determinations were carried out twice in independent experiments and all the inconclusive samples were reanalysed.

5.2.2.1 *CYP2E1*

The *CYP2E1* intronic polymorphism (rs6413432) was determined by PCR-RFLP as described elsewhere (Lin, 1998; Teixeira, 2004) with minor modifications. Primer design and PCR conditions were optimised in order to achieve the best possible results: OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) was used to determine melting temperatures, GC contents, hairpins, and dimmer formation. Basic Local Alignment Search Tool (BLAST) resource (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm low similarity with other human sequences. This polymorphism results in the loss of a restriction site, which allows the common and variant alleles to be discriminated by RFLP after appropriate restriction enzyme digestion. Specific primers, restriction enzyme site details and restriction patterns for each of these SNPs are shown in Table XVII. PCR was performed in a 50 µL reaction volume containing: 100 ng of genomic DNA, 1 µM of each primer, $MgCl_2$ 5 mM, 0.5 U of Immolase (Bioline), 0.2 mM of each dNTP (Bioline) and 2% dimethyl sulfoxide (DMSO), in 1x PCR buffer. The amplification was accomplished in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) and consisted of an initial activation step (7 min at 95 ºC); 35 cycles of denaturation (30 sec at 94 ºC), annealing (30 sec at 62 ºC) and extension (30 sec at 72 ºC); and a final extension step (10 min at 72 ºC). For restriction analysis, 10 µL of the PCR product were digested with 5 units of the *Sex*AI (Fermentas) restriction enzyme (12.5 µl final reaction volume), during 210 min at 37 ºC. After restriction enzyme inactivation (20 min at 65 ºC), restriction fragments were subjected to electrophoresis in

2% agarose gel with ethidium bromide (1 µg/mL) for visualisation under ultraviolet light. HyperLadder IV (Bioline) was used as molecular marker.

5.2.2.2 ***GSTM1* and *GSTT1***

GSTM1 and *GSTT1* genotyping for gene deletions were carried out through a multiplex PCR technique described elsewhere (Lin *et al*., 1998; Teixeira *et al*., 2004) with minor modifications. DNA samples were amplified for *GSTM1* with the primers: 5´-GAA CTC CCT GAA AAG CTA AAG C-3´ (forward) and 5´-GTT GGG CTC AAA TAT ACG GTG G-3´ (reverse) which produced a 219-bp product. For *GSTT1*, DNA samples were amplified with the primers 5´-TCA CCG GAT CAT GGC CAG CA-3´ (forward) and 5´-TTC CTT ACT GGT CCT CAC ATC TC-3´ (reverse) which produced a 459-bp product. For either gene, the success of the amplification procedure depended on the absence of the polymorphic deletion. The amplification of the albumin gene with the primers 5′-GCC CTC TGC TAA CAA GTC CTA C-3′ (forward) and 5′-GCC CTA AAA AGA AAA TCC CCA ATC-3′ (reverse) was used as an internal control and produced a 350-bp product. PCR was performed in a final volume of 50 µL, consisting of 100 ng of genomic DNA, 0.2 mM of each dNTP (Bioline), $MgCl_2$ 5 mM, 0.5 µM of each *GSTM1* primer, 0.15 µM of each *GSTT1* primer, 0.1 µM of each albumin primer, 0.5 U of Immolase (Bioline), 1x PCR buffer and 2% dimethyl sulfoxide (DMSO). Amplification was performed with an initial denaturation at 95 ºC for 7 min, followed by 35 cycles of amplification performed at 94 ºC for 1 min, 62 ºC for 1 min, 72 ºC for 2 min, and a final extension at 72 ºC for 10 min using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). After electrophoretic separation in 2% agarose gel stained with ethidium bromide (1µg/mL), the amplified products were visualised under ultraviolet light.

5.2.2.3 ***GSTP1***

The *GSTP1* (rs1695) polymorphism was determined by PCR-RFLP as described elsewhere (Harries, 1997; Teixeira, 2004), with minor modifications. PCR was performed in a 50 µL reaction volumes containing: 100 ng of genomic DNA, 1 µM of each primer, $MgCl_2$ 10 mM, 0.5 U of Immolase (Bioline), 0.2 mM of each dNTP (Bioline) and 2% dimethyl sulfoxide (DMSO), in 1x PCR buffer. The amplification was accomplished in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) and consisted of an initial activation step (7 min at 95 ºC); 35 cycles of denaturation (1 min at 94 ºC), annealing (1 min at 62 ºC) and extension (1 min at 72 ºC); and a final extension step (10 min at 72 ºC). For restriction analysis, 10 µL of the PCR product were digested with 5 units of the *Bsm*AI (Fermentas) restriction enzyme (12.5 µL final reaction volume),

overnight (16-18 hours) at 37 ºC. After restriction enzyme inactivation (20 min at 65 ºC), restriction fragments were subjected to eletrophoresis in 4% agarose gel with ethidium bromide (1 µg/mL) for visualisation under ultraviolet light. HyperLadder V (Bioline) was used as molecular marker. Primer design and PCR conditions were optimised in order to achieve the best possible results: OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/ OligoAnalyzer/) was used to determine melting temperatures, GC contents, hairpins, and dimmer formation. Basic Local Alignment Search Tool (BLAST) resource (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm low similarity with other human sequences. For *GSTP1* polymorphism the nucleotide change resulted in gain of a restriction site, which therefore allowed the common and variant alleles to be discriminated by RFLP after appropriate restriction enzyme digestion. Detailed information and expected products for each genotype of the tested gene are shown in Table XVII.

5.2.3 **Genotyping of polymorphisms in genes involved in DNA repair**

Detailed information regarding selected SNP's and corresponding base and amino acid exchanges are presented in Table XVI.

5.2.3.1 ***XRCC1, PARP1, MUTYH, RAD51*, *BRIP1* and *FANCA***

XRCC1 (rs1799782 and rs25487), *PARP1* (rs1136410), *MUTYH* (rs3219489), *RAD51* (rs1801321), *BRIP1* (rs4986764) and *FANCA* (rs7190823) were genotyped by real-time PCR, using TaqMan SNP Genotyping Assays (Applied Biosystems). In order to assure uniformity in genomic DNA content (2.5 ng/µL) in all samples, DNA was quantified using the fluorimetric Quant-iT™ Picogreen® dsDNA Assay Kit (Invitrogen) and a Zenyth 3100 plate reader (Anthos Labtech Instruments), according to the manufacturer's recommendations. The PCR amplification was performed in a 7300 Real-Time PCR System thermal cycler (Applied Biosystems), with 96-well microplates containing 10 ng of genomic DNA, 1x SNP Genotyping Assay mix (Applied Biosystems) and 1x TaqMan Universal PCR Master Mix (Applied Biosystems) per well (final volume, 10 µL/well). The specific Genotyping Assay mix used for each polymorphism is identified in Table XVI.

The amplification conditions consisted of an initial enzyme activation step (10 min at 95 ºC), followed by 40 or more amplification cycles consisting of denaturation (15 sec at 92 ºC) and annealing/extension (1 min at 60 ºC). Allelic discrimination was then performed by measuring fluorescence emitted by both VIC and FAM dyes in each well (60 sec) and computing the results into the System SDS software version 1.3.1. The procedure was

repeated for all inconclusive samples. Also, 10-15% of the genotype determinations were carried out twice in independent experiments with 100% of concordance between experiments.

Table XVI. Detailed information on selected SNPs

Gene [a]	Location	db SNP cluster ID (rs no.)	Amino acid change	AB Assay ID
CYP2E1	10q26.3	rs6413432	-- [c]	-- [d]
GSTP1	11q13.2	rs1695	Ile105Val	-- [d]
XRCC1	19q13.2	rs1799782	Arg194Trp	C__11463404_10
XRCC1	19q13.2	rs25487	Gln399Arg	C____622564_10
PARP1	1q41-q42	rs1136410	Val762Ala	C___1515368_1_
MUTYH	1p34.1	rs3219489	Gln335His	C__27504565_10
RAD51	15q15.1	rs1801321	-- [b]	C___7482700_10
XRCC2	7q36	rs3218536	Arg188His	-- [d]
XRCC3	14q32.3	rs861539	Thr241Met	-- [d]
FANCA	16q24.3	rs7190823	Thr266Ala	C__30590701_10
BRIP1	17q22.2	rs4986764	Ser919Pro	C___2547422_10

[a] Deletions at GSTM1 (1p13.3) and GSTT1 (22q11.23) were also analysed but are not considered here, as this table pertains only to SNPs. [b] SNP located on 5' UTR. [c] Intronic SNP. [d] not applicable (genotyping performed by PCR-RFLP).

5.2.3.2 ***XRCC2* and *XRCC3***

XRCC2 (rs3218536) and *XRCC3* (rs861539) polymorphisms were determined by PCR-RFLP as described previously (Bastos *et al*., 2009; Silva *et al*., 2010) with slight modifications. Primer design and PCR conditions were optimised in order to achieve the best possible results: OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) was used to determine melting temperatures, GC contents, hairpins, and dimmer formation. Basic Local Alignment Search Tool (BLAST) resource (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm low similarity with other human sequences. For these polymorphisms the nucleotide change resulted in the gain of a restriction site, which therefore allowed the common and variant alleles to be discriminated by RFLP after appropriate restriction enzyme digestion. Specific primers, restriction enzyme site details and restriction patterns for each of these SNPs are shown in Table XVII. PCR was performed in a 50 µL reaction volumes containing: 100 ng of genomic DNA, 0.6 µM of each primer (Stabvida), $MgCl_2$ 2.5 mM, 0.75 U of Immolase (Bioline) and 0.2 mM of each dNTP (Bioline), in 1.3x PCR buffer. The amplification was accomplished in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) and consisted of an initial activation step (7 min at 95 ºC); 32 (*XRCC2*) or 35 (*XRCC3*) cycles of denaturation (30 sec at 94 ºC), annealing (30 sec at 62 ºC or 64 ºC for *XRCC2*

or *XRCC3*, respectively) and extension (30 sec at 72 ºC); and a final extension step (10 min at 72 ºC). For restriction analysis of the *XRCC2* (rs3218536) polymorphism, 10 μL of the PCR product were digested with 2 units of the *Sex*AI (Fermentas) restriction enzyme (12.5 μL final reaction volume), overnight (16-18 hours) at 37 ºC. For restriction analysis of the *XRCC3* (rs861539) polymorphism, 10 μL of the PCR product were digested with 2.5 units of the *Nla*III (Fermentas) restriction enzyme (12.5 μL final reaction volume), during 180 min at 37 ºC. After restriction enzyme inactivation (20 min at 65 ºC), restriction fragments were subjected to eletrophoresis in 4% agarose gel with ethidium bromide (1 μg/mL) for visualisation under ultraviolet light. HyperLadder V (Bioline) was used as molecular marker. Genotype determinations were carried out twice in independent experiments and all the inconclusive samples were reanalysed.

Table XVII. PCR-RFLP details for the XRCC2 (rs3218536), XRCC3 (rs861539), GSTP1 (rs1695) and CYP2E1 (rs6413432) polymorphisms

SNPs	Primers	PCR product (bp)	SNP effect on restriction enzyme site	Restriction patterns after enzyme digestion
XRCC2	5'-GGT GTA CTG CAG TAG TAG CAC CCA CTT AC-3' (forward) 5'-CAC ATC ACA CAG TCG TCG AGA GGC-3' (reverse)	307	creates a *Sex*AI site	G/G: 307 bp; G/A: 307, 214, 93 bp; A/A: 214, 93 bp.
XRCC3	5'-GTA CTG CTG TCT CGG GGC ATG-3' (forward) 5'-CGA TGG TTA GGC ACA GGC TGC-3' (reverse)	315	creates a *Nla*III site	C/C: 22, 293 bp; C/T: 22, 105, 188, 293 bp; T/T: 22, 105, 188 bp.
GSTP1	5'-ACC CCA GGG CTC TAT GGG AA-3' (forward) 5'-TGA GGG CAC AAG AAG CCC CT-3' (reverse)	176	creates a *Bsm*AI site	A/A: 176 bp; A/G: 85, 91, 176 bp; G/G: 85, 91 bp.
CYP2E1	5'-CTG CTG CTA ATG GTC ACT TG-3' (forward) 5'-GGA GTT CAA GAC CAG CCT AC-3' (reverse)	688	eliminates a *Dra*I site	T/T: 338, 350 bp; T/A: 338, 350, 688 bp; A/A: 688 bp.

5.3 RESULTS

5.3.1 Genotype distribution of polymorphisms in the study population

The genotypic frequencies of polymorphisms in genes involved in the metabolism and DNA repair pathways for sets of exposed workers, controls, and whole population are presented in Tables XVIII and XIX. Moreover, the distribution frequencies of the genotyped polymorphisms in the study population were in agreement with Hardy-Weinberg equilibrium.

Table XVIII. Frequency of genotypes involved in xenobiotic metabolism in the study population

		All		Controls		Exposed		
Gene	*Allele*	***N***	**%**	***N***	**%**	***N***	**%**	***p*-value**
***CYP2E1* intron**								0.552
	T/T	*132*	82.0	*68*	79.1	*64*	85.3	
	T/A	*27*	16.8	*17*	19.8	*10*	13.3	
	A/A	*2*	1.2	*1*	1.2	*1*	1.3	
***GSTM1* deletion**								0.923
	Present	*78*	45.6	*40*	46.0	*38*	45.2	
	Null	*93*	54.4	*47*	54.0	*46*	54.8	
***GSTT1* deletion**								0.626
	Present	*140*	81.9	*70*	80.5	*70*	83.3	
	Null	*31*	18.1	*17*	19.5	*14*	16.7	
***GSTP1* Ile105Val**								0.499
	Ile/ Ile	*69*	40.4	*32*	36.8	*37*	44.0	
	Ile/Val	*84*	49.1	*44*	50.6	*40*	47.6	
	Val/Val	*18*	10.5	*11*	12.6	*7*	8.3	

Between the groups studied, there was no significant difference in the frequency of the genetic polymorphisms of xenobiotic metabolising enzymes. The frequencies of the metabolic polymorphisms found herein are in accordance with previous studies carried out in Caucasians (Garte *et al*., 2001; Laffon *et al*., 2006; López-Cima *et al*., 2012) and related to those already described by other authors on Portuguese populations (Gaspar *et al*., 2004; Teixeira *et al*., 2004; Costa *et al*., 2012).

Regarding the DNA repair genes, there was no significant difference between study groups in the frequency of studied polymorphisms except for *RAD51* 5'UTR and *XRCC2* Arg188His (Table XIX).

The differences observed on genotypic distribution of *RAD51* 5'UTR may be associated to the total number of exposed subjects genotyped compared to controls, whereas for *XRCC2* Arg188His the reason is possibly the rare incidence of the homozygous variant genotype among Caucasians.

The distribution in the study population of the genotypes involved in DNA repair pathways are consistent with those reported by other authors for Caucasian (Figueiroa *et al*., 2007; Frank *et al*., 2007; Wang *et al*., 2009b; Santonocito *et al*., 2012) and more specifically for Portuguese populations (Garcia-Léston *et al*., 2012; Silva *et al*., 2010). Regarding XRCC1, statistical analysis revealed that the two studied genotypes are not in linkage disequilibrium. In another study conducted in a Portuguese population the XRCC1 polymorphisms (Arg194Trp and Gln399Arg) also showed that these loci were not in linkage disequilibrium.

Table XIX. Genotype frequency of genes involved in DNA repair in the study population

		All		Controls		Exposed		
Gene	*Allele*	***N***	**%**	***N***	**%**	***N***	**%**	***p*-value**
***XRCC1* Arg194Trp**[a]								0.058
	Arg/Arg	*151*	91.5	*83*	95.4	*68*	87.2	
	Arg/Trp	*14*	8.5	*4*	4.6	*10*	12.8	
***XRCC1* Gln399Arg**								0.917
	Gln/Gln	*63*	38.2	*32*	36.8	*31*	39.7	
	Gln/Arg	*68*	41.2	*37*	42.5	*31*	397	
	Arg/Arg	*34*	20.6	*18*	20.7	*16*	20.5	
***PARP1* Val762Ala**[a]								0.505
	Val/Val	*141*	85.5	*75*	87.2	*66*	83.5	
	Val/Ala	*24*	14.5	*11*	12.8	*13*	16.5	
***MUTYH* Gln335His**								0.992
	Gln/Gln	*81*	54.0	*45*	53.6	*36*	54.5	
	Gln/His	*55*	36.7	*31*	36.9	*24*	36.4	
	His/His	*14*	9.3	*8*	9.5	*6*	9.1	
***RAD51* 5'UTR**								**0.003**[b]
	G/G	*44*	27.2	*22*	25.6	*22*	28.9	
	G/T	*82*	50.6	*36*	41.9	*46*	60.5	
	T/T	*36*	22.2	*28*	32.6	*8*	10.5	
***XRCC2* Arg188His**								**0.030**[b]
	Arg/Arg	*126*	73.7	*57*	65.5	*69*	82.1	
	Arg/His	*43*	25.0	*28*	32.2	*15*	47.9	
	His/His	*2*	1.2	*2*	2.3	*0*	0	
***XRCC3* Thr241Met**								0.051
	Thr/Thr	*49*	28.8	*24*	27.9	*25*	29.8	
	Thr/Met	*93*	54.1	*42*	48.8	*51*	60.7	
	Met/Met	*28*	16.3	*20*	23.3	*8*	9.5	
***FANCA* Thr266Ala**								0.498
	Thr/Thr	*21*	12.5	*9*	10.5	*12*	14.6	
	Thr/Al	*66*	38.4	*32*	37.2	*34*	41.5	
	Ala/Ala	*81*	47.1	*45*	52.3	*36*	43.9	
***BRIP1* Ser919Pro**								0.146
	Ser/Ser	*16*	9.7	*6*	7.0	*10*	12.7	
	Ser/Pro	*77*	46.7	*46*	53.5	*31*	39.2	
	Pro/Pro	*72*	43.6	*34*	39.5	*38*	48.1	

[a] no variant homozygous
[b] between controls and exposed

5.3.1 **Analysis of association between the genetic polymorphisms and biomarkers studied**

The influence of genetic polymorphisms coding for metabolic and DNA repair enzymes on the level of genotoxicity and lymphocyte subsets are reported in Table XX (only the data with statistically significant or near to significance are shown).

Nuclear buds (BNbud) formation was significantly higher in buccal cells of exposed individuals carrying the *CYP2E1* intron variant allele (A) compared to homozygous wild type individuals. Amongst controls, *CYP2E1* intron genotypes had no effect in the frequency of this biomarker.

Table XX. Influence of biomarkers of susceptibility on genotoxicity parameters (only significant effect are included)

	Controls			Exposed		
	N	**Mean ± SE**	***p*-value**	***N***	**Mean ± SE**	***p*-value**
CYP2E1 intron						
BNbud						
T/T	*53*	0.36 ± 0.077	0.542	*51*	0.80 ± 0.12	**0.022**
T/A+A/A	*15*	0.20 ± 0.11		*7*	1.57 ± 0.20	
GSTM1 deletion						
%TDNA						
Null	*47*	7.66 ± 0.71	0.923	*45*	13.23 ± 0.99	**0.011**
Present	*40*	7.33 ± 0.61		*38*	10.36 ± 0.98	
***GSTT1* deletion**						
Aberrants						
Null	*17*	1.12 ± 0.30	**0.050**	*14*	2.79 ± 0.60	0.649
Present	*70*	2.09 ± 0.22		*70*	3.26 ± 0.32	
GSTP1 Ile105Val						
MNB						
Ile/ Ile	*28*	0.14 ± 0.07	0.729	*29*	0.45 ± 0.11	**0.050**
Ile/Val+Val/Val	*41*	0.20 ± 0.07		*33*	0.82 ± 0.15	
FANCA Thr266Ala						
MNL						
Thr/Thr	*9*	2.33 ± 0.93	0.577	*12*	2.33 ± 0.57	**0.019**
Thr/Ala+ Ala/Ala	*77*	2.84 ± 0.32		*70*	4.74 ± 0.44	

Further differences were found in genotoxicity biomarkers frequencies among exposed workers and control individuals carriers of *GSTM1* and *GSTT1* null alleles, respectively. Indeed, exposed individuals carrying the null *GSTM1* genotype had a significant increase on the percentage of tail DNA (%TDNA) compared to referents expressing the enzyme. In the control group, subjects with the active *GSTT1* genotype showed a near significant increase on aberrant cells frequency compared to null subjects.

The frequency of micronucleus on buccal cells (MNB) was higher in exposed subjects carrying the *GSTP1 Val* allele compared to homozygous wild type; however, the increase was near statistical significance.

Moreover, exposed subjects carrying the *FANCA Ala* variant allele showed a significant increase on micronucleus frequency in peripheral blood lymphocytes (MNL) compared to carriers of the wild type genotype (*Thr/Thr*). No significant differences were found for *FANCA* genotypes among individuals from the control group.

5.4 DISCUSSION

It is generally agreed that exposure assessment and biomonitoring also requires information on the genetic *make-up* of each individual. Given the fact that genetic polymorphisms in metabolic enzymes may cause inter-individual variability in the genotoxic damage induced by xenobiotics, individual risk assessment has to be evaluated by taking into account individual genetics. Hence, genotyping xenobiotic metabolising enzymes, such as cytochrome P450 (CYPs) and glutathione *S*-transferases (GSTs) is of particular importance in risk assessment to hazardous chemicals.

In the present study, we evaluated the impact of major polymorphic metabolising enzymes on the different biomarkers of genotoxicity. Our results showed that metabolic polymorphisms in *CYP2E1, GSTP1* and *GSTM1* genes were able to influence the level of DNA damage induced by FA-exposure (Table XX).

Concerning *CYP2E1* intronic polymorphism, a significant increase on the frequency of BNbud was found in carriers of the variant *A* allele compared to homozygous wild type individuals, suggesting a possible protective effect of the wild-type genotype with regard to genotoxic effects induced by FA inhalation.

As mentioned earlier, the *CYP2E1* enzyme is responsible for the oxidation of various compounds, producing reactive oxygen species (ROS) that can deplete glutathione (Gonzalez, 2007). Buccal mucosa is the first tissue of contact to several agents and probably more susceptible to oxidative stress. The cellular glutathione content is determinant for FA-detoxification since it is the co-factor of the most important enzyme in the metabolic inactivation of FA. Therefore, glutathione depletion may lead to a decrease on FA detoxication and an increase on FA toxicity.

Indeed, *in vitro* experiments showed an increment of FA cytotoxicity with glutathione depletion (Saito *et al*., 2005; Teng *et al*., 2001). Curiously, in glutathione-depleted rat hepatocytes, also inhibited for ADH1 activity, the CYP2E1 inhibitor isoniazid was effective at inhibiting FA metabolism and increasing cytotoxicity, showing that FA can also be a subtract for CYP2E1 enzyme (Teng *et al*., 2001). Data also described ROS formation during cellular treatment with several FA concentrations. Saito *et al*. (2005) reported that FA together with free radicals induced a synergistic increment of the cellular ROS by glutathione depletion that led to an increased cell death, but FA cytotoxicity was associated to cross-link formation.

In conclusion, the increase on cytogenetic damage found in buccal cells of FA-exposed individuals carrying the variant allele may be related to the activity of the encoded CYP2E1 enzyme, probably more effective than the protein expressed by wild type

individuals. The consequent increase of intracellular ROS due to enzyme activity causes glutathione decrease as a result FA detoxication may decrease, thus increasing FA-induced genotoxicity. To our knowledge, this is the first study investigating the influence of *CYP2E1* polymorphism on genotoxicity biomarkers induced by FA-exposure. Therefore, these findings must be cautiously interpreted, as further studies are needed to confirm our results.

With regard to *GSTs* polymorphic genes, our results showed a significant increase of DNA damage, %TDNA, associated with *GSTM1* null allele among FA-exposed workers. This result is not surprising since null individuals, do not express the protein and therefore lacks the enzyme activity. Similarly, subjects carrying *GSTP1* variant *Val* allele had higher frequency of MNB compared to homozygous wild type (p=0.05). The *GSTP1 Val* allele is associated to an enzyme with a lower conjugating activity when compared to the *GSTP1 Ile* allele phenotype (Cote *et al*., 2009). Moreover, *GSTP1* gene polymorphism is associated with bronchial asthma and airway hypersensitivities (Mapp *et al*., 2002; Lee *et al*., 2004), which implicate that *GSTP1* genotypes may be a risk factor for FA exposure. Our results are in accordance with a recent study by Jiang *et al*. (2010) who found non-significant increases on tail moment (comet assay) in FA-exposed subjects carrying the *GSTM1* null genotype and on MNL frequency in *GSTP1* Val allele carriers.

The results found seem to indicate an important role of GSTs enzymes on the genotoxic effect induced by FA exposure, since the lack or diminished function of the enzymes led to an increase of genetic damage in exposed individuals.

GSTs constitute the major defensive antioxidant system against oxidative stress, catalysing the conjugation of glutathione with different species of reactive molecules; their activity, as FA detoxication process is dependent of cell's glutathione content. Some *in vitro* studies indicates that FA can decrease glutathione cellular levels (Teng *et al*., 2001; Zhou *et al*., 2006) suggesting that it may disrupt the intracellular balance between oxidants and antioxidants and cause oxidative stress (Sul *et al*., 2007) through the build-up of ROS levels, observed by Saito *et al*. (2005) and Teng *et al*. (2001). On the other hand, the increase of oxidative stress may activate intracellular signalling pathways leading to increased production of detoxification enzymes such as GSTs.

Our findings together with other authors seems to indicate that the DNA damaged found in null *GSTM1* individuals and carriers of *GSTP1* variant Val allele are not only related to a decrease in the effectiveness of antioxidant defenses but also to oxidative stress induced indirectly by FA exposure, since both processes are glutathione-dependent.

The increase of intracellular ROS may induce oxidative damage to lipids, proteins and DNA (Dalle-Donne *et al*., 2003). Increased levels of malondialdehyde, a biomarker of oxidative stress and lipid peroxidation, was found in the lung tissues of rats exposed to FA (Sul *et al*., 2007). Moreover, in a group of FA- exposed pathologists Bono *et al*. (2010) reported an increase on leukocyte malondialdehyde-deoxyguanosine adducts compared to controls.

A borderline significant increase on aberrant frequencies was found in controls among *GSTT1* positive subjects. This finding is consistent to data indicating that *GSTT1* enzyme have both detoxification and activating activities toward different environmental chemicals (To-Figueiras *et al*., 1997; Raimondi *et al*., 2006), therefore the effect associated with *GSTT1* null allele is difficult to predict.

DNA repair is a very important mechanism in the protection against multiple types of DNA damage, specifically those induced by endogenous and exogenous agents. Common polymorphisms in DNA repair genes may alter protein function and an individual's capacity to repair damaged DNA. A deficient repair capacity may lead to genetic instability and ultimately to cancer initiation.

In the present study, the level of genetic damage induced by FA exposure was significantly affected by *FANCA* genotypes. Subjects carrying the *Ala* variant allele showed a significant increase on MNL frequency compared to those carrying the wild type genotype. This finding suggests that wild-type homozygotes for *FANCA* polymorphism are more proficient to repair efficiently the DNA damage induced by the exposure to FA than the variant allele carriers do.

Our results are in accordance with recent *in vitro* data suggesting a role of FANC repair pathway genes in counteracting FA-induced DNA damage. Chicken DT40 cells deficient in *FANC* genes show a hypersensitive response to treatment with FA (Ridpath *et al*., 2007). Moreover, Rosado *et al*. (2011) reported that DT40 cells with *FANC* knockouts are synthetic lethal with mutations in the FA-catabolism *ADH5* gene, suggesting that FA-cytotoxicity, evidenced by increased CAs, is generated when it is accumulated in the cell.

FANCA integrates the FANC repair pathway as a subunit of the FANC core complex. This complex comprises eight FANC proteins required to activate the FANC repair system, to resolve DNA crosslinks and to stabilise stalled replication forks. The phosphorylation of the FANCA protein seems to be an important step in the response of the FANC pathway to damaged DNA (Collins *et al*., 2009). Additionally, recent *in vitro* data indicates FANCA as one of the proteins involved in the stabilisation of stalled replication forks induced by

hydroxyurea treatment. The absence of functional protein led to an increase in chromosomal instability, evidenced by chromosomal aberrations (Schlacher *et al*., 2012).

In addition to the present investigation, only a few studies were carried on FA exposed populations to understand the influence of genetic polymorphisms of DNA repair and xenobiotic-metabolising enzymes on observed genetic damage. Some studies have reported positive outcomes, while others have not. A recent study found a significant association between carriers of the variant allele Met for *XRCC3* Thr241Met and BNbud (Ladeira *et al*., 2013). However, in our study this effect was only observed for metabolic genes and not for *XRCC3* Thr241Met. Jiang *et al*. (2006) reported significant differences on DNA damage for *XRCC1* Arg280His; subjects with the variant genotype showed an increase on comet tail length compared to wild-type homozygotes. In contrast, the increase on CAs found in a group of pathologists was not affected by *GSTM1* or *GSTT1* null genotypes (Santovito *et al*., 2011). However, the result may have been influenced by the low number of individuals included in the study, since in a previous study, with a smaller population, we also did not find any association between *GSTM1*, *GSTT1* and the damage induced by FA-exposure (Costa *et al*., 2008).

Lastly, our results indicate that polymorphisms in DNA repair and xenobiotic metabolising enzymes may affect an individual response to the DNA damage induced by occupational exposure to FA, evidenced in both buccal cells and lymphocytes. An important association was found for *CYP2E1*, *GSTM1*, *GSTP1* and *FANCA* polymorphic genes. It is likely that a combined action of enzymatic detoxication and DNA repair is needed to protect cells against FA effects.

Variability in the expression and/or activity of enzymes is attributed to a number of sources, including genetic variation. Nevertheless, the contributions of genetics are controversial. In many cases, genotype does not correlate well with phenotype, a phenomenon which is likely due to the fact that these enzymes are also induced to varying degrees by external factors (lifestyle factors, drugs, stress) (Peck and Eaton, 2010).

III. General Conclusions

"All substances are poisons; there is none which is not a poison.

The right dose differentiates a poison from a remedy."

Paracelsus (1493–1541)

The current labour force stands about 45% of the world's population. Their work sustains the economic and material basis of society which is critically dependent on their working capacity. Thus, health at work and healthy work environments are one of the most valuable assets of individuals, communities and countries. Hence, a policy-oriented research focused on the development of integrated approaches regarding the health risk assessment of populations occupationally exposed to hazard compounds is of paramount importance.

In anatomical pathology laboratories, Formaldehyde (FA) is a well-known compound routinely used for over 100 years as a fixative and tissue preservative. Because of its sensitizing properties, irritating effects and cancer implication, FA accounts probably for the most important chemical-exposure hazard concerning this professional group.

Indeed data from the present study shows that subjects working in anatomical pathology laboratories are regularly exposed to average levels of FA higher (0.38 ppm) than national and international limit values, which indicates a potential risk to worker's health.

Our findings also suggest that the level of formic acid in urine can be used to estimate the occupational exposure to FA in a group of workers, since its measurement effectively indicates the exposure to FA.

Regarding the genotoxicity evaluation, all cytogenetic and DNA damage endpoints were significantly elevated in the peripheral blood lymphocytes (PBLs) of pathological anatomy professionals exposed to FA (0.38 ppm) compared to control subjects. Our findings reinforce recent data indicating the biological plausibility of inhaled FA to induce genotoxic damage on circulating blood cells and potentially on other distant-site cells. Furthermore, the same biological effect, higher frequency of micronucleus formation, observed in these surrogate cells also occurs in FA-target cells from buccal mucosa. The association found between the micronucleus formation in these two tissues and FA-levels of exposure and duration, confirms this biomarker sensitivity to evaluate the genotoxic action of FA in occupational exposed subjects.

Chromosome breakage (measured by CAs and comet assay) in PBLs was significantly higher in FA-exposed workers compared to controls, which may suggest a clastogenic

mode of action of FA, and the primary mechanism of micronucleus formation. Moreover, the increase on SCEs frequency among workers seems to reflect the repair of FA-induced DNA lesions by homologous repair (HR) pathway.

Concerning the immunological markers, our findings indicate that FA exposure may affect the percentage of lymphocyte subpopulations. Moreover, this influence seems to be associated with immunosuppression. To our knowledge, this is the first European study evaluating these indicators in FA-exposed professionals from anatomical pathology laboratories. Therefore, these results must be cautiously interpreted, until further replication in other studies. In addition, the activity of the different subpopulations was not measured in this study.

As evident from the literature, various genetic polymorphisms may modulate the incidence of various diseases, particularly cancer, as well as genotoxic effects induced by occupational exposure to xenobiotics. In theory, studies on individual susceptibility should allow us to establish various favourable and adverse genotypes in order to minimise the exposure risk in sensitive individuals. In this study, we found interesting associations between metabolic polymorphisms in *CYP2E*1, *GSTP1* and *GSTM1* genes and the level of DNA damage induced by FA exposure. Our results also reveal a potential novel repair pathway involved in the repair of genetic lesions caused by FA-occupational exposure, the FANC repair pathway. The level of genetic damage induced by FA exposure was significantly affected by *FANCA* genotypes. This finding suggests the inclusion of the FANC pathway in further human biomonitoring studies to explore potential individual susceptibility to FA and other genotoxic agents.

The present results show the importance of individual susceptibility factors in modulating genotoxicity, although cautious interpretations are required since the size of the studied population limits the power of many of the analysis. Because the effects of these polymorphisms are relatively subtle, and some important alleles are relatively rare, a much larger study population will be necessary to evaluate their effects on biomarkers, especially when gene-gene interactions are considered.

Overall, our data indicates a potential health risk situation of anatomical pathology laboratory workers exposed to FA (0.38 ppm). Thus, implementation of security and hygiene measures may be crucial to decrease the risk associated to FA occupational exposure. Substitution of the chemical for one less hazardous is generally the first hazard control measure considered, since it is usually the less expensive and the most positive method for controlling hazards. However, in FA's case because it is a long-established material, effective and inexpensive, few compounds can replace it without compromising

quality and cost, which delays its substitution in anatomo-pathology procedures by both producers and users.

Control and minimisation of the exposure are the next step to protect workers from the high levels of airborne FA in the workplace. This may be achieved by engineering control measures, including the installation of equipment that allows an effective general ventilation and local (benches) exhaust, a control of the room temperature and ventilated sample cabinets or rooms. In addition, the use of appropriate masks to protect from FA vapours, may be determinant to reduce exposure in some tasks where communication is not required, for instance, during the disposal of waste solutions. Note that this task can be countered by hiring specialised companies for waste disposal solutions of formalin, which would eliminate a major source of exposure. Other crucial measures to control and reduce the risk to FA exposure in anatomical pathology laboratories are regular monitoring and surveillance activities, including, periodic air sampling and medical surveillance, as well as good practice campaigns, training programs and implementation of written policies and procedures.

> "With respect to formaldehyde or any other "toxin of the moment, education and prevention are essential to minimizing the risk of becoming embroiled in "bet-the-company" litigation."
>
> *Excerpt from an advertising brochure of Thompson Hine, a top US business law firm.*

IV. REFERENCES

Alan, D., and D'Andrea, M.D. 2010. The Fanconi Anemia and Breast Cancer Susceptibility Pathways. New England Journal of Medicine, 362(20):1909-19.

Alli, B.O. 2008. Fundamental Principles of Occupational Health and Safety. International Labour Office (ILO), Geneva, 200 pp.

Aalto-Korte, K., Kuuliala, O., Suuronen, K., and Alanko, K. 2008. Occupational contact allergy to formaldehyde and formaldehyde releasers. Contact Dermatitis, 59(5):280-9.

Abbas, A.K., and Lichtman, A.H. 2004. Células y tejidos del sistema inmunitario. In: Immunología celular y molecular. 5ª edición. Elsevier, Madrid, 16-39 pp.

ACGIH (American Conference of Governmental Industrial Hygienists). 2013. TLV's and BEI's – Based on the Documentation of the Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices. ACGIH, 238 pp.

AFSSET (L'Agence française de sécurité sanitaire de l'environnement et du travail). 2008. Valeurs limites d'exposition en milieu professionnel. Évaluation des effets sur la santé et des methods de mesure des niveaux d'exposition sur le lieu de travail pour le formaldehyde. Maisons-Alfort, France, 62 pp.

Albertini, R.J., Nicklas, J.A., and O'Neill, J.P. 1993. Somatic cell gene mutations in humans: biomarkers for genotoxicity. Environmental Health Perspectives, 101(Suppl.3):193-201.

Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E., Tice, R., Waters, M.D., Aitio, A. 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. Mutation Research, 463(2): 111-72.

Almeida Santos, M.F., Ferrari, I. and Luna, H. 2005. Chromosomal aberration analysis in workers exposed to chemical and biological hazards in research laboratories. Environmental Research, 97(3):330-4.

Almeida, K.H., and Sobol, R.W. 2007. A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification. DNA Repair (Amst), 6(6):695-711.

Aka, P., Mateuca R., Kirsch-Volders M. 2008. DNA repair enzyme genotypes and their toxicologically relevant phenotypes. In: State of art of genotype vs. phenotype studies. Nofer Institute of Occupational Medicine. Lodz, Poland, 47-57 pp.

Akbar-Khanzadeh, F., Vaquerano, M.U., Akbar-Khanzadeh, M., and Bisesi, M.S. 1994. Formaldehyde exposure, acute pulmonary response, and exposure control options in a gross anatomy laboratory. American Journal of Industrial Medicine, 26(1):61-75.

Akbar-Khanzadeh, F. and Pulido, E.V. 2003. Using Respirators and Goggles to Control Exposure to Air Pollutants in an Anatomy Laboratory. American Journal Industrial Medicine, 43:326-331.

Akiyama, M., Kyoizumi, S., Hirai, Y., Kusunoki, Y., Iwamoto, K.S., and Nakamura, N. 1995. Mutation frequency in human blood cells increases with age. Mutation Research, 338(1-6):141-9.

Anderson, L.N., Cotterchio, M., Mirea, L., Ozcelik, H., and Kreiger, N. 2012. Passive cigarette smoke exposure during various periods of life, genetic variants, and breast cancer risk among never smokers. American Journal of Epidemiology, 175(4):289-301.

Andreu-Ballester, J.C., García-Ballesteros, C., Benet-Campos, C., Amigó, V., Almela-Quilis, A., Mayans, J., and Ballester, F. 2012. Values for αβ and γδ T-lymphocytes and CD4+, CD8+, and CD56+ subsets in healthy adult subjects: assessment by age and gender. Cytometry Part B Clinical Cytometry, 82(4):238-44.

Anzenbacher, P., and Anzenbacherová E. 2001. Cytochromes P450 and metabolism of xenobiotics. Cellular and Molecular Life Sciences, 58(5-6):737-47.

Aquilina, G., and Bignami, M. 2001. Mismatch repair in correction of replication errors and processing of DNA damage. Journal of Cellular Physiology, 187(2):145-54.

Armstrong, R.W., Imrey, P.B., Lye, M.S., Armstrong, M.J., Yu, M.C. and Sani, S. 2000. Nasopharyngeal carcinoma in Malaysian Chinese: Occupational exposures to particles, formaldehyde and heat. International Journal of Epidemiology, 29, 991–998.

Arts, J.H., Rennen, M.A., and de Heer, C. 2006. Inhaled formaldehyde: evaluation of sensory irritation in relation to carcinogenicity. Regulatory Toxicology and Pharmacology, 44(2):144-60.

ATSDR (Agency for Toxic Substances and Disease Registry). 1999. Toxicological Profile for formaldehyde. US Department of Health and Human Services, Public Health Service, Atlanta GA, USA, 421 pp.

ATSDR (Agency for Toxic Substances and Disease Registry). 2010. Addendum to the Toxicological Profile for formaldehyde. US Department of Health and Human Services, Public Health Service, Atlanta GA, USA, 144 pp.

Au, W.W., Salama, S.A., and Sierra-Torres, C.H. 2003. Functional characterization of polymorphisms in DNA repair genes using cytogenetic challenge assays. Environmental Health Perspectives, 111(15):1843-50.

Au, W.W. 2007. Usefulness of biomarkers in population studies: from exposure to susceptibility and to prediction of cancer. International Journal of Hygiene and Environmental Health, 210(3-4):239-46.

Autrup, H. 2000. Genetic polymorphisms in human xenobiotica metabolizing enzymes as susceptibility factors in toxic response. Mutation Research, 464: 65–76.

Aydin, S., Canpinar, H., Ündeğer, Ü., Güç, D., Çolakoğlu, M., Kars, A., and Başaran N. 2013. Assessment of immunotoxicity and genotoxicity in workers exposed to low concentrations of formaldehyde. Archives of Toxicology, 87(1):145-53.

Azevedo, F., Marques, F., Fokt, H., Oliveira, R., and Johansson, B. 2011. Measuring oxidative DNA damage and DNA repair using the yeast comet assay. Yeast, 28(1):55-61.

Azqueta, A., Meier, S., Priestley, C., Gutzkow, K.B., Brunborg, G., Sallette, J., Soussaline, F., and Collins, A. 2011a. The influence of scoring method on variability in results obtained with the comet assay. Mutagenesis, 26(3):393-9.

Azqueta, A., Gutzkow, K.B., Brunborg, G., and Collins, A.R. 2011b. Towards a more reliable comet assay: optimising agarose concentration, unwinding time and electrophoresis conditions. Mutation Research, 724(1-2):41-5.

Bach, B., Pedersen, O.F., and Mohvave, L. 1990. Human Performance During Experimental Formaldehyde Exposure, Environmental International 16:105-113 [abstract].

Bachand, A.M., Mundt, K.A., Mundt, D.J., and Montgomery, R.R. 2010. Epidemiological studies of formaldehyde exposure and risk of leukemia and nasopharyngeal cancer: a meta-analysis. Critical Reviews in Toxicology, 40(2):85-100.

Ballarin, C., Sarto, F., Giacomelli, L., Bartolucci, G.B., and Clonfero, E. 1992. Micronucleated cells in nasal mucosa of formaldehyde-exposed workers. Mutation Research, 280(1): 1-7.

Balmain, A., Gray, J., and Ponder, B. 2003. The genetics and genomics of cancer. Nature genetics, 33 Suppl:238-44.

Banno, K., Kisu, I., Yanokura, M., Tsuji, K., Masuda, K., Ueki, A., Kobayashi, Y., Yamagami, W., Nomura, H., Tominaga, E., Susumu, N., Aoki, D. 2012. Epimutation and cancer: a new carcinogenic mechanism of Lynch syndrome. International Journal of Oncology, 41(3):793-7.

Barroso, E., Pita, G., Arias, J.I., Menendez, .P, Zamora, P., Blanco, M., Benitez, J., and Ribas, G. 2009. The Fanconi anemia family of genes and its correlation with breast cancer susceptibility and breast cancer features. Breast Cancer Research and Treatment, 118(3):655-60.

Bastos, H.N., Antão, M.R., Silva, S.N., Azevedo, A.P., Manita, I., Teixeira, V., Pina, J.E., Gil, O.M., Ferreira, T.C., Limbert, E., Rueff, J., and Gaspar, J.F.2009. Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk. Thyroid, 19(10):1067-75.

Bauchinger, M., and Schmid, E. 1985. Cytogenetic effects in lymphocytes of formaldehyde workers of a paper factory. Mutation Research, 158(3):195-9.

Beane Freeman, L.E., Blair, A., Lubin, J.H., Stewart, P.A., Hayes, R.B., Hoover, R.N., and Hauptmann, M. 2009. Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries: the National Cancer Institute Cohort. Journal of the National Cancer Institute, 101(10):751-61.

Beane Freeman, L.E., Blair, A., Lubin, J.H., Stewart, P.A., Hayes, R.B., Hoover, R.N., and Hauptmann, M. 2013. Mortality from solid tumors among workers in formaldehyde industries: an update of the NCI cohort. American Journal of Industrial Medicine, 56(9):1015-26

Benhamou, S., Lee, W.J., Alexandrie, A.K., Boffetta, P., Bouchardy, C., Butkiewicz, D., Brockmöller, J., Clapper, M.L., Daly, A., Dolzan, V., Ford, J., Gaspari, L., Haugen, A., Hirvonen, A., Husgafvel-Pursiainen, K., Ingelman-Sundberg, M., Kalina, I., Kihara, M., Kremers, P., Le Marchand, L., London, S.J., Nazar-Stewart, V., Onon-Kihara, M., Rannug, A., Romkes, M., Ryberg, D.,Seidegard, J., Shields, P., Strange, R.C., Stücker, I., To-Figueras, J., Brennan, P., and Taioli, E. 2002. Meta- and pooled analyses of the effects of glutathione S-transferase M1 polymorphisms and smoking on lung cancer risk. Carcinogenesis, 23(8):1343-50.

Berode, M., Sethre, T., Läubli, T., and Savolainen, H. 2000. Urinary methanol and formic acid as indicators of occupational exposure to methyl formate. International Archives of Occupational and Environmental Health, 73(6):410-4.

Biró, A., Pállinger, E., Major, J., Jakab, M. G., Klupp, T., Falus, A., and Tompa, A. 2002. Lymphocyte phenotype analysis and chromosome aberration frequency of workers occupationally exposed to styrene, benzene, polycyclic aromatic hydrocarbons or mixed solvents. Immunology Letters, 81: 133-140.

Bisset, L.R., Lung, T.L., Kaelin, M., Ludwig, E., and Dubs, R.W. 2004. Reference values for peripheral blood lymphocyte phenotypes applicable to the healthy adult population in Switzerland. European Journal of Haematology, 72(3):203-12.

Bin, Q., and Luo, J. 2013. Role of polymorphisms of GSTM1, GSTT1 and GSTP1 Ile105Val in Hodgkin and non-Hodgkin lymphoma risk: a Human Genome Epidemiology (HuGE) review. Leukemia and Lymphoma, 54(1):14-20.

Binetti, R., Costamagna, F.M., and Marcello, I. 2006. Development of carcinogenicity classifications and evaluations: the case of formaldehyde. Annali dell'Istituto superiore di sanità, 42(2):132-43.

Blair, A., Zheng, T., Linos, A., Stewart, P.A., Zhang, Y.W., Cantor, K.P.2001. Occupation and leukemia: a population-based case-control study in Iowa and Minnesota. American Journal of Industrial Medicine, 40(1):3-14.

Bloching, M., Hofmann, A., Lautenschläger, C., Berghaus, A., Grummt, T. 2000. Exfoliative cytology of normal buccal mucosa to predict the relative risk of cancer in the upper aerodigestive tract using the MN-assay. Oral Oncology, 36(6):550-5.

Boccia, S., De Lauretis, A., Gianfagna, F., van Duijn, C.M., and Ricciardi, G. 2007. CYP2E1PstI/RsaI polymorphism and interaction with tobacco, alcohol and GSTs in gastric cancer susceptibility: A meta-analysis of the literature. Carcinogenesis, 28(1):101-6.

Boeniger, M.F. 1987. Formate in urine as a biological indicator of formaldehyde exposure: a review. American Industrial Hygiene Association Journal, 48(11):900-908.

Bolognesi, C., Abbondandolo, A., Barale, R., Casalone, R., Dalprà, L., De Ferrari, M., Degrassi, F., Forni, A., Lamberti, L., Lando, C., Migliore, L., Padovani, P., Pasquini, R., Puntoni, R., Sbrana, I., Stella, M., and Bonassi, S. 1997. Age-related increase of baseline frequencies of sister chromatid exchanges, chromosome aberrations, and micronuclei in human lymphocytes. Cancer Epidemiology Biomarkers and Prevention, 6(4):249-56.

Bolognesi, C., Lando, C., Forni, A., Landini, E., Scarpato, R., Migliore, L., and Bonassi S. 1999. Chromosomal damage and ageing: effect on micronuclei frequency in peripheral blood lymphocytes. Age Ageing, 28(4): 393-7.

Bolognesi, C., and Fenech, M. 2013. Micronucleus assay in human cells: lymphocytes and buccal cells. Methods Molecular Biology,1044:191-207.

Bolt, H.M., Roos, P.H., and Their, R. 2003. The cytochrome P-450 isoenzyme CYP2E1 in the biological processing of industrial chemicals: consequences for occupational and environmental medicine. International Archives Occupational Environmental Health, 76(3):174-85.

Bonassi, S., Fenech, M., Lando, C., Lin, Y.P., Ceppi, M., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Jia, C., Di Giorgio, M., Ferguson, L.R., Fucic, A., Lima, O.G., Hrelia, P., Krishnaja, A.P., Lee, T.K., Migliore, L., Mikhalevich, L., Mirkova, E., Mosesso, P., Müller, W.U., Odagiri, Y., Scarffi, M.R., Szabova, E., Vorobtsova, I., Vral, A., and Zijno A. 2001. HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. Environmental and Molecular Mutagenesis, 37(1):31-45.

Bonassi, S., and Au W.W. 2002. Biomarkers in molecular epidemiology studies for health risk prediction. Mutation Research, 511(1):73-86.

Bonassi, S., Ugolini, D., Kirsch-Volders, M., Strömberg, U., Vermeulen, R., Tucker, J.D. 2005. Human population studies with cytogenetic biomarkers: review of the literature and future prospectives. Environmental and Molecular Mutagenesis, 45: 258–70.

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A.,Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., and Fenech, M. 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis, 28(3):625-31.

Bonassi, S., Coskun, E., Ceppi, M., Lando, C., Bolognesi, C., Burgaz, S., Holland, N., Kirsh-Volders, M., Knasmueller, S., Zeiger, E., Carnesoltas, D., Cavallo, D., da Silva, J., de Andrade, V.M., Demircigil, G.C., Domínguez O.A., Donmez-Altuntas, H., Gattas, G., Giri, A., Giri, S., Gómez-Meda, B., Gómez-Arroyo, S., Hadjidekova, V., Haveric, A., Kamboj, M., Kurteshi, K., Martino-Roth, M.G., Montero, M.R., Nersesyan, A., Pastor-Benito, S., Favero S.D.M., Shaposhnikova, A., Stopper, H., Thomas, P., Torres-Bugarín, O.,Yadav, A.S., Zúñiga-González G., and Fenech, M. 2011. The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN(XL): the role of life-style, host factors, occupational exposures, health status, and assay protocol. Mutation Research, 728(3):88-97.

Bono, R., Romanazzi, V., Munnia, A., Piro, S., Allione, A., Ricceri, F., Guarrera, S., Pignata, C., Matullo, G., Wang, P., Giese, R.W., and Peluso, M. 2010. Malondialdehyde-deoxyguanosine adduct formation in workers of pathology wards: the role of air formaldehyde exposure. Chemical Research in Toxicology, 23(8):1342-8.

Bosetti, C., McLaughlin, J.K., Tarone, R.E., Pira, E., and La Vecchia, C. 2008. Formaldehyde and cancer risk: a quantitative review of cohort studies through 2006. Annals of Oncology, 19(1):29-43.

Boscolo, P., Di Gioacchino, M., Sabbioni, E., Benvenuti, F., Conti, P., Reale, M., Bavazzano, P., and Giuliano, G. 1999. Expression of lymphocyte subpopulations, cytokine serum levels, and blood and urinary trace elements in asymptomatic atopic men exposed to an urban environment. International Archives of Occupational and Environmental Health, 72: 26-32.

Boucharat, C., Desauziers, V., and Le Cloirec, P. 1998. Experimental design for the study of two derivatization procedures for simultaneous GC analysis of acidic herbicides and water chlorination by-products. Talanta, 47 (2): 311–323.

Bourner, R.D., Parry, E.M., and Parry, J.M. 1998. Chemically induced aneuploidy: investigations into chromosome specific effects in mitosis. Mutation Research, 404(1-2):191-7.

Brennan, P., Lewis, S., Hashibe, M., Bell, D.A., Boffetta, P., Bouchardy, C., Caporaso, N., Chen, C., Coutelle, C., Diehl, S.R., Hayes, R.B., Olshan, A.F., Schwartz, S.M., Sturgis, E.M., Wei, Q., Zavras, A.I, and Benhamou, S. 2004. Pooled analysis of alcohol dehydrogenase genotypes and head and neck cancer: a HuGE review. American Journal Epidemiology, 159(1):1-16

Brevik, A., Galvão, I., Medin, T., Jørgenesen, A., Piasek, A., Elilasson, J., Karlsen, A., Blomhoff, R., Veggan, T., Duttaroy, A.K., and Collins, A.R. 2011a. Supplementation of a western diet with golden kiwifruits (*Actinidia chinensis var*.'Hort 16A':) effects on biomarkers of oxidation damage and antioxidant protection. Nutrition Journal, 10:54.

Brevik, A., Karlsen, A., Azqueta, A., Tirado, A.E., Blomhoff, R., and Collins, A. 2011b. Both base excision repair and nucleotide excision repair in humans are influenced by nutritional factors. Cell Biochemistry and Function, 29(1):36-42.

Burgaz, S., Cakmak, G., Erdem, O., Yilmaz, M., and Karakaya, A.E. 2001. Micronuclei frequencies in exfoliated nasal mucosa cells from pathology and anatomy laboratory workers exposed to formaldehyde. Neoplasma, 48 (2): 144-147.

Burgaz, S., Erdem, O., Cakmak, G., Erdem, N., Karakaya, A., and Karakaya, A.E. 2002. Cytogenetic analysis of buccal cells from shoe-workers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde. Biomarkers, 7(2): 151-161.

Butkiewicz, D., Grzybowska, E., Phillips, D.H., Hemminki, K., and Chorazy M. 2000. Polymorphisms of the GSTP1 and GSTM1 genes and PAH-DNA adducts in human mononuclear white blood cells. Environmental and Molecular Mutagenesis, 35(2):99-105.

Cai, Z., Chen, H., Tao, J., Guo, W., Liu, X., Zheng, B., Sun, W., and Wang, Y. 2012. Association of base excision repair gene polymorphisms with ESRD risk in a Chinese population. Oxidative Medicine and Cellular Longevity, 2012:928421.

Cantor, S., Drapkin, R., Zhang, F., Lin, Y., Han, J., Pamidi, S., Livingston, D.M. 2004. The BRCA1-associated protein BACH1 is a DNA helicase targeted by clinically relevant inactivating mutations. Proceedings of the National Academy of Sciences of the United States of America, 101(8):2357-62.

Cantor, S.B., Bell, D.W., Ganesan, S., Kass, E.M., Drapkin, R., Grossman, S., Wahrer, D.C., Sgroi, D.C., Lane, W.S., Haber, D.A., and Livingston, D.M. 2001. BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. Cell, 105(1):149-60.

Cantor, S.B., and Guillemette, S. 2011. Hereditary breast cancer and the BRCA1-associated FANCJ/BACH1/BRIP1. Future Oncology, 7(2):253-61.

Carbonell, E., Peris, F., Xamena, N., Creus, A., and Marcos, R. 1996. Chromosomal aberration analysis in 85 control individuals. Mutation Research, 370(1):29-37.

Caria, H., Chaveca, T., Laires, A., and Rueff, J. 1995. Genotoxicity of quercetin in the micronucleus assay in mouse bone marrow erythrocytes, human lymphocytes, V79 cell line and identification of kinetochore-containing (CREST staining) micronuclei in human lymphocytes. Mutation Research, 343: 85–94

Cariaso, M., and Lennon, G. 2012. SNPedia: a wiki supporting personal genome annotation, interpretation and analysis. Nucleic Acids Research, 40(Database issue):D1308-12.

Carrano, A., and Natarajan, A., 1988. Considerations for population monitoring using cytogenetic techniques. Mutation Research: Human monitoring (special issue), 204: 379-406.

Casanova-Schmitz, M., David, R.M., and Heck, H.D. 1984. Oxidation of formaldehyde and acetaldehyde by NAD+-dependent dehydrogenases in rat nasal mucosal homogenates. Biochemical Pharmacology, 33(7):1137-42

Casset, A., Marchand, C., Purohit, A., le Calve, S., Uring-Lambert, B., Donnay, C., Meyer, P., and de Blay, F. 2006. Inhaled formaldehyde exposure: effect on bronchial response to mite allergen in sensitized asthma patients. Allergy. 61(11):1344-50.

Castella, M., Pujol, R., Callén, E., Trujillo, J.P., Casado, J.A., Gille, H., (…) Barbot, J., Tapia, M., Molinés, A., Figuera, A., Bueren, J.A., and Surrallés, J. 2011. Origin, functional role, and clinical impact of Fanconi anemia FANCA mutations. Blood, 117(14):3759-69.

Cederbaum, AI. 2006. CYP2E1—biochemical and toxicological aspects and role in alcohol induced liver injury. The Mount Sinai Journal of Medicine, 73:657–672.

Ceppi, M., Biasotti, B., Fenech, M., and Bonassi, S. 2010. Human population studies with the exfoliated buccal micronucleus assay: statistical and epidemiological issues. Mutation Research, 705(1):11-9.

Chambers, G.K., Marshall, S.J., Robinson, G.M., Maguire, S., Newton-Howes, J., Chong, N.L. 2002. The genetics of alcoholism in Polynesians: alcohol and aldehyde dehydrogenase genotypes in young men. Alcoholism, Clinical and Experimental Research, 26: 949–55.

Chandra, R.K.1999. Nutrition and immunology: from the clinic to cellular biology and back again. The Proceedings of the Nutrition Society, 58(3):681-683.

Chang, J.S., Wrensch, M.R., Hansen, H.M., Sison, J.D., Aldrich, M.C., Quesenberry, C.P. Jr, Seldin, M.F., Kelsey, K.T., Wiencke, J.K. 2009. Base excision repair genes and risk of lung cancer among San Francisco Bay Area Latinos and African-Americans. Carcinogenesis, 30(1):78-87.

Chen, Z., Lou, J., Chen, S., Zheng, W., Wu, W., Jin, L., Deng, H., and He, J. 2006. Evaluating the genotoxic effects of workers exposed to lead using micronucleus assay, comet assay and TCR gene mutation test. Toxicology, 223(3):219-26.

Cheng, G., Wang, M., Upadhyaya, P., Villalta, P.W., and Hecht, S.S. 2008a. Formation of formaldehyde adducts in the reactions of DNA and deoxyribonucleosides with alpha-acetates of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and N- nitrosodimethylamine (NDMA). Chemical Research in Toxicology, 21(3): 746-751.

Cheng, X.M., Zhao, J., Feng, B., Wen, P.E., and Shao, H. 2008b. Relationship between susceptibility of formaldehyde metabolism and genetic polymorphisms of ALDH2 and cytochrome P4502E1. Zhonghua Yu Fang Yi Xue Za Zhi 42(8):582-7. [abstract]

CDC (Centers for Disease Control and Prevention). 2008 (amended in 2010). Final Report on Formaldehyde Levels in FEMA-Supplied Travel Trailers, Park Models, and Mobile Homes. CDC, Atlanta, USA, 58 pp.

Chaganti, R.S., Schonberg, S., and German, J. 1974. A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proceedings of the National Academy of Sciences of the United States of America, 71(11):4508-12.

Chng, W.C., Tan, G.B., and Kuperan, P. 2004. Establishment of adult peripheral blood lymphocyte subset reference range for an Asian population by single platform flow cytometry: Influence of age, sex and race and comparison with other published studies. Clinical and Diagnostic Laboratory Immunology, 11: 168-173.

Cho, E.Y., Hildesheim, A., Chen, C.J., Hsu, M.M., Chen, I.H., Mittl, B.F., Levine, P.H., Liu, M.Y., Chen, J.Y., Brinton, L.A., Cheng, Y.J., and Yang, C.S. 2003. Nasopharyngeal carcinoma and genetic polymorphisms of DNA repair enzymes XRCC1 and hOGG1. Cancer Epidemiology, Biomarkers and Prevention, 12:1100-1104.

Cho, H.J., Lee, S.Y., Ki, C.S., and Kim, J.W. 2005. GSTM1, GSTT1 and GSTP1 polymorphisms in the Korean population. Journal of Korean Medical Science, 20(6):1089-1092.

Clevers, H., Alarcon, B., Wileman, T., and Terhorst, C. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. Annual Review of Immunology, 6:629-662.

Coelho, P., García-Lestón, J., Silva, S., Costa, C., Costa, S., Coelho, M., Laffon, B., Pásaro, E., and Teixeira, J.P. 2011. Geno- and Immunotoxic effects on populations living near a mine - Case study: Panasqueira mine. Journal of Toxicology and Environmental Health Part A, 74: 1076-1086.

Coggon, D., Harris, E.C., Poole, J., and Palmer, K.T. 2003. Extended follow-up of a cohort of British chemical workers exposed to formaldehyde. Journal of the National Cancer Institute, 95(21):1608-1615.

Collins, A.R. 1996. DNA damage and its repair-Molecular and Cellular Genetics. Principles of Medical Biology volume 5, 229-254 pp.

Collins, J.J., Acquavella, J.F., and Esmen, N.A. 1997. An updated meta-analysis of formaldehyde exposure and upper respiratory tract cancers. Journal Occupational Environmental Medicine, 39(7):639-51.

Collins, B.H., Horská, A., Hotten, P.M., Riddoch, C., and Collins, A.R. 2001. Kiwifruit protects against oxidative DNA damage in human cells and in vitro. Nutrition and Cancer, 39(1):148-53.

Collins, A.R., Harrington, V., Drew, J., and Melvin, R. 2003. Nutritional modulation of DNA repair in a human intervention study. Carcinogenesis, 24(3):511-5.

Collins, A.R. 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Molecular Biotechnology, 26(3):249-61.

Collins, J.J. and Lineker, G.A. 2004. A review and meta-analysis of formaldehyde exposure and leukemia. Regulatory Toxicology and Pharmacology, 40, 81-91.

Collins, N.B., Wilson, J.B., Bush, T., Thomashevski, A., Roberts, K.J., Jones, N.J., and Kupfer, G.M. 2009. ATR-dependent phosphorylation of FANCA on serine 1449 after DNA damage is important for FA pathway function. Blood, 113(10):2181-90.

Colosio, C., Corsini, E., Barcellini, W., and Maroni, M. 1999. Immune parameters in biological monitoring of pesticide exposure: current knowledge and perspectives. Toxicology Letters, 108(2-3):285-95.

Compton, P.J., Hooper, K., and Smith, M.T. 1991. Human somatic mutation assays as biomarkers of carcinogenesis. Environmental Health Perspectives, 94:135-41.

Conaway, C.C., Whysner, J., Verna, L.K., and Williams, G.M. 1996. Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. Pharmacology and Therapeutics, 71(1-2):29-55.

Constantinou, A. 2012. Rescue of replication failure by Fanconi anaemia proteins. Chromosoma, 121(1):21-36.

Cortés-Gutiérrez, EI., Dávila-Rodríguez, M.I., Fernández, J.L., López-Fernández, C.,Gosálbez, A., and Gosálvez, J. 2011. New application of the comet assay: chromosome—comet assay. Journal of Histochemistry and Cytochemistry, 59(7):655-60.

Costa, C. 2012. Pesticides and health in agriculture. Genetic Damage. PhD Thesis, Instituto de Ciências Biomédicas Abel Salazar (ICBAS), 169 pp.

Costa, C., Costa, S., Silva, S., Coelho, P., Botelho, M., Gaspar, J., Rueff, J., Laffon, B., and Teixeira JP. 2012. DNA damage and susceptibility assessment in industrial workers exposed to styrene. Journal of Toxicology and Environmental Health Part A, 75(13-15):735-46.

Costa, C., Teixeira, J.P., Silva, S., Roma-Torres, J., Coelho, P., Gaspar, J., Alves, M., Laffon, B., Rueff, J., and Mayan, O. 2006. Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. Mutagenesis, 21(5):343-50.

Costa, S., Coelho, P., Costa, C., Silva, S., Mayan, O., Santos, L.S, Gaspar, J.and Teixeira, J.P. 2008. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. Toxicology, 252(1-3): 40-48.

Costa, S., Pina, C., Coelho, P., Costa, C., Silva, S., Porto, B., Laffon, B. and Teixeira, J.P. 2011. Occupational exposure to formaldehyde: genotoxic risk evaluation by comet assay and micronucleus test using human peripheral lymphocytes. Journal of Toxicology and Environmental Health Part A, 74(15-16): 1040-1051.

Costa S., Brandão F., Coelho M., Costa C., Coelho P., Silva S., Porto B., and Teixeira J.P. 2013. Micronucleus frequencies in lymphocytes and buccal cells in formaldehyde exposed workers. WIT Transactions on Biomedicine and Health (Environmental Health Risk, EHR 2013), 16: 83-94.

Cote, M.L., Chen, W., Smith, D.W., Benhamou, S., Bouchardy, C., Butkiewicz, D., Tokudome, S., Pu, Y., Yang, P., Wenzlaff, A.S., Wikman, H., and Taioli E. 2009. Meta- and pooled analysis of GSTP1 polymorphism and lung cancer: a HuGE-GSEC review. American Journal of Epidemiology, 169(7):802-14.

Cotelle, S., and Férard, J.F. 1999. Comet assay in genetic ecotoxicology: a review. Environmental and Molecular Mutagenesis, 34(4):246-55.

Cui, X., Brenneman, M., Meyne, J., Oshimura, M., Goodwin, E.H., and Chen, D.J. The XRCC2 and XRCC3 repair genes are required for chromosome stability in mammalian cells. Mutation Research, 434(2):75-88.

D'Agostini, F., Balansky, R.M., Izzotti, A., Lubet, R.A., Kelloff, G.J., and De Flora, S. 2001. Modulation of apoptosis by cigarette smoke and cancer chemopreventive agents in the respiratory tract of rats. Carcinogenesis, 22(3):375-80.

Dhawan, A., Bajpayee, M., and Parmar, D. 2009. Comet assay: a reliable tool for the assessment of DNA damage in different models. Cell Biology and Toxicology, 25(1):5-32.

Dallas, C.E., Scott, M.J., Ward, J.B. Jr, and Theiss, J.C. 1992. Cytogenetic analysis of pulmonary lavage and bone marrow cells of rats after repeated formaldehyde inhalation. Journal of Applied Toxicology, 12(3):199-203 [abstract].

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, and Colombo R. 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clinical Chimica Acta, 329(1-2):23-38.

Davies, A.A., Masson, J.Y., McIlwraith, M.J., Stasiak, A.Z., Stasiak, A., Venkitaraman, A.R., and West, S.C. 2001. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. Molecular Cell, 7(2):273-82.

DeMarini, D.M. 2004. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutation Research, 567(2-3):447-74.

Descotes, J. 2004. Classification of immunotoxicants. In Immunotoxicology of drugs and chemicals: an experimental approach. Volume I: Principles and methods of immunotoxicology. Amsterdam, Elsevier, 379-385 pp.

Ding, X., and Kaminsky, L.S. 2003. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. Annual Review of Pharmacology and Toxicology, 43:149-73.

Doseth, B., Visnes, T., Wallenius, A., Ericsson, I., Sarno, A., Pettersen, H.S., Flatberg, A., Catterall, T., Slupphaug, G., Krokan, H.E., and Kavli, B. 2011. Uracil-DNA glycosylase in base excision repair and adaptive immunity: species differences between man and mouse. Journal of Biological Chemistry, 286(19):16669-80.

Duthie, S.J., Jenkinson, A.M., Crozier, A., Mullen, W., Pirie, L., Kyle, J., Yap, L.S., Christen, P., and Duthie, G.G. 2006. The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. European Journal of Nutrition, 45(2):113-22.

Edling, C., Hellquist, H., and Odkvist, L. 1988. Occupational exposure to formaldehyde and histopathological changes in the nasal mucosa. British Journal Industrial Medicine, 45(11):761-5.

Egan, K.M., Cai, Q., Shu, X.O., Jin, F., Zhu, T.L., Dai, Q., Gao, Y.T., and Zheng, W. 2004. Genetic polymorphisms in GSTM1, GSTP1, and GSTT1 and the risk for breast cancer: results from the Shanghai Breast Cancer Study and meta-analysis. Cancer Epidemiology, Biomarkers and Prevention, 13(2):197-204.

Emri, G., Schaefer, D., Held, B., Herbst, C., Zieger, W., Horkay, I., Bayerl, C. 2004. Low concentrations of formaldehyde induce DNA damage and delay DNA repair after UV irradiation in human skin cells. Experimental Dermatology, 13(5):305-15.

Engel, L.S., Taioli, E., Pfeiffer, R., Garcia-Closas, M., Marcus, P.M., Lan, Q., Boffetta, P., Vineis, P., Autrup, H., Bell, D.A., Branch, R.A., Brockmöller, J., Daly, A.K., Heckbert, S.R., Kalina, I., Kang, D., Katoh, T., Lafuente, A., Lin, H.J., Romkes, M., Taylor, J.A., and Rothman, N. 2002. Pooled analysis and meta-analysis of glutathione S-transferase M1 and bladder cancer: a HuGE review. American Journal of Epidemiology, 156(2):95-109.

Ersson, C., Møller P.; Forchhammer, L.; Loft, S., Azqueta, A., Godschalk, R.W.L., van Schooten, F.J., Jones, G.D., Higgins, J.A., Cooke, M.S., Mistry, V., Karbaschi, M., Phillips, D.H., Sozeri O, Routledge, M.N., Nelson-Smith, K., Riso, P., Porrini, M., Matullo, G., Allione, A., Stepnik, M., Ferlińska, M., Teixeira, J.P., Costa, S., Corcuera, L.A. López de Cerain, A., Laffon, B., Valdiglesias., V., Collins, A., and Möller, L. 2013. An ECVAG inter-

laboratory validation study of the comet assay: inter-laboratory and intra-laboratory variation of DNA strand breaks and FPG-sensitive sites in human mononuclear cells. Mutagenesis, 28: 279–286.

EU (European union). 2013. Commission Decision 2013/204/EU. Official Journal of the European Union L 117 (ISS 1977-0677), 56:18-19.

Eurachem Working Group.1998. The fitness for purpose of analytical methods a Laboratory guide to method validation and related topics. Eurachem, UK, 61 pp.

Ezratty, V., Bonay, M., Neukirch, C., Orset-Guillossou, G., Dehoux, M., Koscielny, S., Cabanes, P.A., Lambrozo, J., and Aubier, M. 2007. Effect of formaldehyde on asthmatic response to inhaled allergen challenge. Environmental Health Perspectives, 115(2):210-4.

Fagnani E., Melios C.B., Pezza L., Pezza H.R. 2003. Chromotropic acid–formaldehyde reaction in strongly acidic media. The role of dissolved oxygen and replacement of concentrated sulphuric acid. Talanta, 60(1): 171–176.

Fang, X., and Zhang, P. 2011. Aneuploidy and tumorigenesis. Seminars in cell and Developmental biology, 22(6):595-601.

Fenech, M. 1993. The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. Environmental Health Perspectives, 101: 101-7.

Fenech, M. 1997. The advantages and disadvantages of the cytokinesis-block micronucleus method. Mutation Research, 392(1-2):11-8.

Fenech, M. 1998. Important variables that influence base-line micronucleus frequency in cytokinesis-blocked lymphocytes-a biomarker for DNA damage in human populations. Mutation Research, 404(1-2):155-65.

Fenech, M. 2006. Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. Mutation Research, 600(1-2):58-66.

Fenech, M. 2007. Cytokinesis-block micronucleus cytome assay. Nature Protocols, 2:1084–1104.

Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J.W., Parry, J., Norppa, H., Eastmond, D.A., Tucker, J.D., and Thomas, P. 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis, 26(1):125-32.

Feron, V.J., Bruyntjes, J.P., Woutersen, R.A., Immel, H.R., and Appelman, L.M. 1988. Nasal tumours in rats after short-term exposure to a cytotoxic concentration of formaldehyde. Cancer Letters, 39 (1):101-11.

Ferro A., Ponte A., Silva A., Viegas C., Quintino F., Albuquerque P., Fernandes S., Viegas S. 2005. A qualidade do ar interior nos serviços de anatomia patológica. Revista Segurança, 167: 45-50.

FIOH (Finnish Institute of Occupational Health). 1998. CAREX- international information system on occupational exposure to known and suspected carcinogens in 1990-93. European Union, Helsinki.

http://www.ttl.fi/en/chemical_safety/carex/countries/portugal/pages/default.aspx.

Figueroa JD, Malats N, Real FX, Silverman D, Kogevinas M, Chanock S, Welch R, Dosemeci M, Tardon A, Serra C. 2007. Genetic variation in the base excision repair pathway and bladder cancer risk. Human Genetics, 121(2):233–42.

Forchhammer, L., Johansson, C., Loft, S., Möller, L., Godschalk, R., Langie, S., Jones, G.D., Kwok, R.W., Collins, A.R., Azqueta, A., Phillips, D.H., Sozeri, O., Stepnik, M., Palus, J., Vogel, U., Wallin, H., Routledge, M.N., Handforth, C., Allione, A., Matullo, G., Teixeira, J.P., Costa, S., Riso, P., Porrini, M., and Møller, P. 2010. Variation in the measurement of DNA damage by comet assay measured by the ECVAG inter-laboratory validation trial. Mutagenesis, 25: 113-123.

Forchhammer, L., Ersson, C., Loft, S., Möller, L., Godschalk, R.W.L., Jones, G.D., Higgins, J.A., Cooke, M., Mistry, V., Karbaschi, M., Collins, A.R., Azqueta, A., Phillips, D.H., Sozeri, O., Routledge, M.N., Nelson-Smith, K., Riso, P., Porrini, M., Matullo, G., Allione, A., Stępnik, M., Komorowska, M., Teixeira, J.P., Costa, S., Corcuera, L.A., López de Cerain, A., Laffon, B., Valdiglesias. V., and Møller P. 2012. Inter-laboratory variation in DNA damage using a standard comet assay protocol. Mutagenesis, 27: 665-672.

Frank B, Hemminki K, Meindl A, Wappenschmidt B, Sutter C, Kiechle M, Bugert P, Schmutzler RK, Bartram CR, Burwinkel B. 2007. BRIP1 (BACH1) variants and familial breast cancer risk: a case-control study. BMC Cancer, 7:83.

Franks, S.J. 2005. A mathematical model for the absorption and metabolism of formaldehyde vapour by humans. Toxicology Applied Pharmacology, 206(3):309-20.

Fransman, W., McLean, D., Douwes, J., Demers, P.A., Leung, V., and Pearce, N. Respiratory symptoms and occupational exposures in New Zealand plywood mill workers. Annals of Occupational Hygiene, 47(4):287–295.

Frenzilli, G., Bosco, E., and Barale, R. 2000. Validation of single cell gel assay in human leukocytes with 18 reference compounds. Mutation Research, 468(2): 93-108.

Gabriel, H.E., Crott, J.W., Ghandour, H., Dallal, G.E., Choi, S.W., Keyes, M.K., Jang, H., Liu, Z., Nadeau, M., Johnston, A., Mager, D., and Mason, J.B. 2006. Chronic cigarette smoking is associated with diminished folate status, altered folate form distribution, and increased genetic damage in the buccal mucosa of healthy adults. American Journal of Clinical Nutrition, 83(4):835-41.

Gaffney, S.H., and Paustenbach, D.J. A proposed approach for setting occupational exposure limits for sensory irritants based on chemosensory models. Annals of Occupational Hygiene. 51(4):345-56.

Garaycoechea, J.I., Crossan, G.P., Langevin, F., Daly, M., Arends, M.J., Patel, K.J. 2012. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. Nature, 489:571-575.

García-Dabrio, M.C., Pujol-Moix, N., Martinez-Perez, A., Fontcuberta, J., Souto, J.C., Soria, J.M., and Nomdedéu, J.F. 2012. Influence of age, gender and lifestyle in lymphocyte subsets: report from the Spanish Gait-2 Study. Acta Haematologica, 127(4):244-9.

García-Lestón, J., Roma-Torres, J., Vilares, M., Pinto, R., Cunha, L.M., Prista, J., Teixeira, J.P., Mayan, O., Pásaro, E., Méndez, J., Laffon, B. 2011. Biomonitoring of a population of Portuguese workers exposed to lead. Mutation Research, 721(1):81-8.

García-Lestón, J., Roma-Torres, J., Vilares, M., Pinto, R., Prista, J., Teixeira, J.P., Mayan, O., Conde, J., Pingarilho, M., Gaspar, J.F., Pásaro, E., Méndez, J., and Laffon, B. 2012. Genotoxic effects of occupational exposure to lead and influence of polymorphisms in genes involved in lead toxicokinetics and in DNA repair. Environmental International, 43: 29-36.

Garte, S., Gaspari, L., Alexandrie, A.K., Ambrosone, C., Autrup, H., Autrup, J.L., Baranova, H., Bathum, L., Benhamou, S., Boffetta, P., Bouchardy, C., Breskvar, K., Brockmoller, J., Cascorbi, I., Clapper, M.L., Coutelle, C., Daly, A., Dell'Omo, M., Dolzan, V., Dresler, C.M., Fryer, A., Haugen, A., Hein, D.W., Hildesheim, A., Hirvonen, A., Hsieh, L.L., Ingelman-Sundberg, M., Kalina, I., Kang, D., Kihara, M., Kiyohara, C., Kremers, P., Lazarus, P., Le Marchand, L., Lechner, M.C., van Lieshout, E.M., London, S., Manni, J.J., Maugard, C.M., Morita, S., Nazar-Stewart, V., Noda, K., Oda, Y., Parl, F.F., Pastorelli, R., Persson, I., Peters, W.H., Rannug, A., Rebbeck, T., Risch, A., Roelandt, L., Romkes, M., Ryberg, D., Salagovic, J., Schoket, B., Seidegard, J., Shields, P.G., Sim, E., Sinnet, D., Strange, R.C., Stücker, I., Sugimura, H., To-Figueras, J., Vineis, P., Yu, M.C., and Taioli

E. 2001. Metabolic gene polymorphism frequencies in control populations. Cancer Epidemiology Biomarkers and Prevention, 10(12):1239-48.

Garrett, M.H., Hooper, M.A., Hooper, B.M., Rayment, P.R., and Abramson, M.J. 1999. Increased risk of allergy in children due to formaldehyde exposure in homes. Allergy, 54, 330–337.

Gaspar, J., Rodrigues, S., Gil, O.M., Manita, I., Ferreira, T.C., Limbert, E., Gonçalves, L., Pina, J.E., and Rueff, J. 2004. Combined effects of glutathione S-transferase polymorphisms and thyroid cancer risk. Cancer Genetics Cytogenetics, 151: 60-67.

Gasser, S., and Raulet, D.H. 2006. The DNA damage response arouses the immune system. Cancer Research, 66(8):3959-62.

Georgieva, A.V., Kimbell, J.S., and Schlosser, P.M. 2003. A distributed-parameter model for formaldehyde uptake and disposition in the rat nasal lining. Inhalation Toxicology, 15(14):1435-63.

Golden, R. 2011. Identifying an indoor air exposure limit for formaldehyde considering both irritation and cancer hazards. Critical Reviews in Toxicology, 41(8):672-721

Goode, E.L., Ulrich, C.M., and Potter, J.D. 2002. Polymorphisms in DNA repair genes and associations with cancer risk. Cancer Epidemiology, Biomarkers and Prevention, 11(12):1513-30.

Gonzalez FJ. 2007. The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. Drug Metabolism and Disposition, 35(1):1-8.

Gottschling, L.M., Beaulieu, H.J., and Melvin, W.W. 1984. Monitoring of formic acid in urine of humans exposed to low levels of formaldehyde. American Industrial Hygiene Association Journal, 45(1):19-23.

Goud, K.I., Hasan, Q., Balakrishna, N., Rao, K.P., and Ahuja, Y.R. 2004. Genotoxicity evaluation of individuals working with photocopying machines. Mutation Research, 563(2):151-8.

Gresner, P., Gromadzinska, J., Polanska, K., Twardowska, E., Jurewicz, J., and Wasowicz, W. 2012. Genetic variability of Xrcc3 and Rad51 modulates the risk of head and neck cancer. Gene, 504(2):166-74.

Griffin, C.S., Simpson, P.J., Wilson, C.R., and Thacker, J. 2000. Mammalian recombination-repair genes XRCC2 and XRCC3 promote correct chromosome segregation. Nature Cell Biology, 2(10):757-61.

Grzegorzewska, A.E., and Leander M. 2005. Total lymphocyte count and subpopulation lymphocyte counts in relation to dietary intake and nutritional status of peritoneal dialysis patients. Advances in peritoneal dialysis, 21:35-40.

Gullo, C., Low, W.K., and Teoh, G. 2008. Association of Epstein-Barr virus with nasopharyngeal carcinoma and current status of development of cancer-derived cell lines. Annals of the Academy of Medicine Singapore, 37(9):769-77.

Guo, Y.M., Wang, Q., Liu, Y.Z., Chen, H.M., Qi, Z., and Guo, Q.H. 2008. Genetic polymorphisms in cytochrome P4502E1, alcohol and aldehyde dehydrogenases and the risk of esophageal squamous cell carcinoma in Gansu Chinese males. World Journal of Gastroenterology, 14(9):1444-9.

Hagmar, L., Bonassi, S., Strömberg, U., Brøgger, A., Knudsen, L.E., Norppa, H., and Reuterwall, C. 1998. Chromosomal aberrations in lymphocytes predict human cancer: a report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). Cancer Research, 58(18):4117-21.

Hagmar, L., Strömberg, U., Bonassi, S., Hansteen, I.L., Knudsen, L.E., Lindholm, C., and Norppa, H. 2004. Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts.Cancer Research, 64(6):2258-63.

Harries, L.W., Stubbins, M.J., Forman, D., Howard, G.C., and Wolf, C.R. 1997. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. Carcinogenesis, 18(4):641-4.

Hansen, J., and Olsen, J.H. 1995. Formaldehyde and cancer morbidity among male employees in Denmark. Cancer Causes Control, 6(4):354-60.

Hayes, J.D., and Pulford, D.J. 1995. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Critical Reviews in Biochemistry and Molecular Biology, 30(6):445-600.

Hayes, R.B., Blair, A., Stewart, P.A., Herrick, R.F., and Mahar, H. 1990. Mortality of U.S. embalmers and funeral directors. American Journal of Industrial Medicine, 18(6):641-52.

Hauptmann, M., Lubin, J.H., Stewart, P.A., Hayes, R.B., and Blair, A. 2003. Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries. J Natl Cancer Inst 95(21): 1615-1623.

Hauptmann, M., Lubin, J.H., Stewart, P.A., Hayes, R.B. and Blair, A. 2004. Mortality from solid cancers among workers in formaldehyde industries. American Journal of Epidemiol., 159, 1117–1130.

Hauptmann, M., Stewart, P.A., Lubin, J.H., Beane Freeman, L.E., Hornung, R.W., Herrick, R.F., Hoover, R.N., Fraumeni, J.F. Jr, Blair, A., and Hayes, R.B. 2009. Mortality from lymphohematopoietic malignancies and brain cancer among embalmers exposed to formaldehyde. Journal of the National Cancer Institute, 101(24):1696-708.

HCN (Health Council of the Netherlands). 2000. Committee on Updating of Occupational Exposure Limits. Health-based Reassessment of Administrative Occupational Exposure Limits. Formic acid; The Hague: Health Council of the Netherlands, publication no. 2000/15OSH ISBN 90-5549-350-3 .

He, J.L., Jin, L.F., and Jin, H.Y. 1998. Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay. Biomedical and Environmental Sciences, 11(1):87-92.

Heck, Hd., and Casanova, M. 2004. The implausibility of leukemia induction by formaldehyde: a critical review of the biological evidence on distant-site toxicity. Regulatory Toxicology and Pharmacology, 40(2):92-106.

Heinzow B., Ellrott T. 1992. Formic acid in urine - a significant parameter in environmental diagnosis. Zentralbl Hyg Umweltmed, 192(5): 455-461 [abstract].

Helleday, T. 2003. Pathways for mitotic homologous recombination in mammalian cells. Mutation Research, 532(1-2):103-15.

Hemminki, K., and Shields, P.G. 2002. Skilled use of DNA polymorphisms as a tool for polygenic cancers. Carcinogenesis, 23:379–80.

Herausgegeben, von A.S., Bernauer, U., Madle, S., Mielke, H., Herbst, U., Richter-Reichhelm, H.B., Appel, K.E., and Gundert-Remy U. 2006. Bundesinstitut für Risikobewertung (BfR) - Assessment of the Carcinogenicity of Formaldehyde [CAS No. 50-00-0]. Berlin, Germany, 156 pp.

Herber, R. F. M., Duffus, J.H., Christensen, J. M., Olsen, E. and Park, M.V. 2001. Risk assessment for occupational exposure chemicals. A review of current methodology. Pure and Applied Chemistry, 73:993-1031.

Hernandez-Castro, B., Doniz-Padilla, L. M., Salgado-Bustamante, M., Rocha, D., Ortiz-Perez, M. D., Jimenez-Capdeville, M. E., Portales-Pérez, D. P., Quintanar-Stephano, A., and González-Amaro, R. 2009. Effect of arsenic on regulatory T cells. Journal of Clinical Immunology, 29: 461-469.

High, K.P. 2001. Nutritional strategies to boost immunity and prevent infection in elderly individuals. Clinical Infectious Diseases, 33(11):1892-900.

Hildesheim, A., Dosemeci, M., Chan, C.C., Chen, C.J., Cheng, Y.J., Hsu, M.M., Chen, I.H., Mittl, B.F., Sun, B., Levine, P.H., Chen, J.Y., Brinton, L.A., and Yang, C.S. 2001. Occupational exposure to wood, formaldehyde, and solvents and risk of nasopharyngeal carcinoma. Cancer Epidemiology, Biomarkers and Prevention, 10(11):1145-53.

Hirvonen, A. 2008. State of art of genotype vs. phenotype studies. Nofer Institute of Occupational medicine. Lodz, Poland, 98 pp.

Hirvonen, A., Godschalk, R., and Kirsch-Volders, M. 2008. Phase II enzyme genotypes and their toxicologically relevant phenotypes. In: State of art of genotype vs. phenotype studies. Nofer Institute of Occupational medicine. Lodz, Poland, 47-57 pp.

Hiyama, T., Yoshihara, M., Tanaka, S., and Chayama, K. 2008. Genetic polymorphisms and head and neck cancer risk (Review). International Journal Oncology, 32(5):945-73.

Hoelzl, C., Knasmüller, S., Misík, M., Collins, A., Dusinská, M., and Nersesyan, A. 2009. Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: recent results and trends. Mutation Research, 681(1):68-79.

Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., and Fenech, M. 2008. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutation Research, 659(1-2):93-108.

Hosgood, H. D. 3rd, Zhang, L., Tang, X., Vermeulen. R., Hao, Z., Shen, M., Qiu, C., Ge, Y., Hua, M., Ji, Z., Li, S., Xiong, J., Reiss, B., Liu, S., Xin, K.X., Azuma, M., Xie, Y., Freeman, L.B., Ruan, X., Guo, W., Galvan, N., Blair, A., Li, L., Huang, H., Smith, M.T., Rothman, N., and Lan, Q. 2012. Occupational exposure to formaldehyde and alterations in lymphocyte subsets. American Journal of Industrial Medicine, 56(2):252-7.

HSE (Health and Safety Executive). 2011. EH40/2005 (second edition, published 2011) Workplace exposure limits. Containing the list of workplace exposure limits for use with the Control of Substances Hazardous to Health Regulations (as amended), London, UK, 74 pp.

Huang, M., Dinney, C.P., Lin, X., Lin, J., Grossman, H.B. and Wu, X. 2007. High-order interactions among genetic variants in DNA base excision repair pathway genes and smoking in bladder cancer susceptibility. Cancer Epidemiology Biomarkers and Prevention, 16: 84-91.

Hung, R.J., Hall, J., Brennan, P., and Boffetta P. 2005. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. American Journal of Epidemiology, 162(10):925-42.

IARC (International Agency for Research on Cancer). 2004. IARC monographs on the evaluation of carcinogenic risks to humans, Volume 83: Tobacco smoke and involuntary smoking. World Health Organization, Lyon, France, 1451 pp.

IARC (International Agency for Research on Cancer). 2006. IARC Monographs on the Evaluation of Carcinogenic Risks to Human, Volume 88: Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxy-2-propanol. World Health Organization, Lyon, France, 39-325 pp.

IARC (International Agency for Research on Cancer). 2012. A Review of Human Carcinogens: Chemical Agents and Related Occupations, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Part F of Volume 100. World Health Organization, Lyon, France, 401-435 pp.

Iarmarcovai, G., Sari-Minodier, I., Orsière, T., De Méo, M., Gallice, P., Bideau, C., Iniesta, D., Pompili, J., Bergé-Lefranc, J.L., and Botta, A. 2006. A combined analysis of XRCC1, XRCC3, GSTM1 and GSTT1 polymorphisms and centromere content of micronuclei in welders. Mutagenesis, 21(2):159-65.

Iarmarcovai, G., Bonassi, S., Botta, A., Baan, R.A., and Orsière, T. 2008. Genetic polymorphisms and micronucleus formation: a review of the literature. Mutation Research, 658(3):215-33.

IHS Chemical. 2012. Chemical Economics Handbook- Formaldehyde. http: //www.ihs.com/products/chemical/planning/ceh/formaldehyde.aspx [abstract]. (consulted on 17th of November, 2013)

Im H., Oh E., Mun J., Khim J.Y., Lee E., Kang H.S., Kim E., Kim H., Won N.H., Kim Y.H., Jung W.W., Sul D. 2006. Evaluation of toxicological monitoring markers using proteomic analysis in rats exposed to formaldehyde. Journal of Proteome Research, 5(6): 1354-66.

Ioannides, C. 2007. Bioactivation of chemicals by cytochromes P450. Environmental Biotechnology, 3 (1):1-9.

IPCS (International Programme on Chemical Safety).1989. Environmental Health Criteria 89: Formaldehyde. World Health Organization, Geneva, Switzerland, 219 pp.

IPCS (International Programme on Chemical Safety). 2002. Concise international chemical assessment document (CICAD); no. 40 - Formaldehyde. World Health Organization, Geneva, Switzerland, 74 pp.

IPQ (Instituto Português da Qualidade). 2007. NP-1796: Segurança e Saúde do Trabalho, Valores limite de exposição profissional a agentes químicos. IPQ, Portugal, 53 pp.

Iversen, O.H. 1986. Formaldehyde and skin carcinogenesis. Environmental International, 12:541–544.

Jacquemont, C. and Taniguchi, T. 2006. Disruption of the fanconi anemia pathway in human cancer in the general population. Cancer Biology and Therapy, 5:1637 – 1639.

Jakab, M.G., Klupp, T., Besenyei, K., Biró, A., Major, J., and Tompa, A. 2010. Formaldehyde-induced chromosomal aberrations and apoptosis in peripheral blood lymphocytes of personnel working in pathology departments. Mutation Research, 698(1-2):11-7.

Janeway CA Jr, Travers P, Walport M. 2001. Immunobiology: The Immune System in Health and Disease, 5th edition edited by Garland Science, New York. US. http://www.ncbi.nlm.nih.gov/books/NBK27098/

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., and Forman, D. 2011. Global cancer statistics. CA: Cancer Journal Clinicians, 61(2):69-90.

Jentsch-Ullrich, K., Koenigsmann, M., Mohren, M., and Franke, A. 2005. Lymphocyte subsets' reference ranges in an age- and gender-balanced population of 100 healthy adults--a monocentric German study. Clinical Immunology, 116(2):192-7.

Jeppesen, D.K., Bohr, V.A., and Stevnsner, T. 2011. DNA repair deficiency in neurodegeneration. Progress in Neurobiolology, 94(2):166-200.

Ji, Z., Li, X., Fromowitz, M., Mutter-Rottmayer, E., Tung, J., Smith, M.T., Zhang, L. 2014. Formaldehyde induces micronuclei in mouse erythropoietic cells and suppresses the expansion of human erythroid progenitor cells. Toxicology Letters, 224(2):233-9.

Jiang, S.F., Yu, L.Q., Leng, S.G., Zhang, Y.S., Cheng, J., Dai, Y.F., Niu, Y., He, F,S,, and Zheng, Y.X. 2006. Association between XRCC1 gene polymorphisms and DNA damage of workers exposed to formaldehyde. Wei Sheng Yan Jiu, 35(6):675-7 [abstract].

Jiang, W., Lu, Y., Chen, Z., Chen, S., Zhang, M., Jin, L., Lou, J., and He, J. 2008. Studying the genotoxicity of vincristine on human lymphocytes using comet assay, micronucleus assay and TCR gene mutation test in vitro. Toxicology, 252(1-3):113-7.

Jiang, S., Yu, L., Cheng, J., Leng, S., Dai, Y., Zhang, Y., Niu, Y., Yan, H., Qu, W., Zhang, C., Zhang, K., Yang, R., Zhou, L., Zheng, Y. 2010. Genomic damages in peripheral blood lymphocytes and association with polymorphisms of three glutathione S-transferases in workers exposed to formaldehyde. Mutation Research, 695(1-2):9-15.

Just, W., Zeller, J., Riegert, C., and Speit, G. 2011. Genetic polymorphisms in the formaldehyde dehydrogenase gene and their biological significance. Toxicology Letters, 207(2):121-7.

Kamata, E., Nakadate, M., Uchida, O., Ogawa, Y., Suzuki, S., Kaneko, T., Saito, M., and Kurokawa, Y. 1997. Results of a 28-month chronic inhalation toxicity study of formaldehyde in male Fisher-344 rats. Journal of Toxicological Sciences, 22(3):239-54.

Karahalil, B., Karakaya, A.E., and Burgaz, S. 1999. The micronucleus assay in exfoliated buccal cells: application to occupational exposure to polycyclic aromatic hydrocarbons. Mutation Research, 442(1):29-35.

Kassie, F., Parzefall, W., and Knasmüller, S. 2000. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. Mutation Research, 463: 13-31.

Keil CB, Akbar-Khanzadeh F, Konecny KA. 2001. Characterizing formaldehyde emission rates in a gross anatomy laboratory. Applied Occupational Environmental Hygiene, 16(10):967-72.

Kennedy, R.D., and D'Andrea, A.D. 2005. The Fanconi Anemia/BRCA pathway: new faces in the crowd. Genes and Development, 19(24):2925-40.

Kerns, W.D., Pavkov, K.L., Donofrio, D.J., Gralla, E.J., and Swenberg, J.A. 1983. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Research, 43(9):4382-92.

Kim, C.W., Song, J.S., Ahn, Y.S., Park, S.H., Park, J.W., Noh, J.H., and Hong, C.S. 2001. Occupational asthma due to formaldehyde. Yonsei Medical Jounal, 42(4):440-5.

Kim, H., and D'Andrea, A.D. 2012. Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. Genes and Development, 26(13):1393-408.

Kim, E.M., Lee, H.Y., Lee, E.H., Lee, K.M., Park, M., Ji, K.Y., Jang, J.H., Jeong, Y.H., Lee, K.H., Yoon, I.J., Kim, S.M., Jeong, M.J., Kim, K.D., Kang, H.S. 2013. Formaldehyde exposure impairs the function and differentiation of NK cells. Toxicology Letters, 223(2):154-61.

Kimbell, J.S., Overton, J.H, Subramaniam, R.P., Schlosser, P.M., Morgan, K.T., Conolly, R.B., and Miller, F.J. 2001. Dosimetry modeling of inhaled formaldehyde: binning nasal flux predictions for quantitative risk assessment. Toxicological Sciences, 64(1):111-21.

Kitaeva, L.V., Mikheeva, E.A., Shelomova, L.F., and Shvartsman, PIa. 1996. Genotoxic effect of formaldehyde in somatic human cells in vivo. Genetika, 32(9):1287-90 [abstract].

Khanna, K.K., and Jackson, S.P. 2001. DNA double-strand breaks: signaling, repair and the cancer connection. Nature Genetics, 27(3):247-54.

Kiyohara, C., and Yoshimasu, K. 2007. Genetic polymorphisms in the nucleotide excision repair pathway and lung cancer risk: a meta-analysis. International Journal of Medical Sciences, 4(2):59-71.

Koivusalo, M., Baumann, M., and Uotila, L. 1989. Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase. FEBS Letters, 257(1):105-9

Kongruttanachok, N., Sukdikul, S., Setavarin, S., Kerekhjanarong, V., Supiyaphun, P., Voravud, N., Poovorawan, Y., and Mutirangura, A. 2001. Cytochrome P450 2E1 polymorphism and nasopharyngeal carcinoma development in Thailand: a correlative study. BMC Cancer, 1:4.

Koleva, V.P., Dragoeva, A.P., Andreeva, AI., Burova, M.T., Georgiev, S., Enchev, D.D. 2013. Comparative analysis of clastogen-induced chromosome aberrations observed with light microscopy and by means of atomic force microscopy. Mutation Research, 753(1):29-35.

Konopacka, M. 2003. Effect of smoking and aging on micronucleus frequencies in human exfoliated buccal cells. Neoplasma, 50(5):380-2.

Kote-Jarai, Z., Jugurnauth, S., Mulholland, S., Leongamornlert, D.A., Guy, M., Edwards, S., Tymrakiewitcz, M., O'Brien, L., Hall, A., Wilkinson, R., Al Olama, A.A., Morrison, J., Muir, K., Neal, D., Donovan, J., Hamdy, F., Easton, D.F., Eeles, R; UKGPCS Collaborators and British Association of Urological Surgeons' Section of Oncology. 2009. A recurrent truncating germline mutation in the BRIP1/FANCJ gene and susceptibility to prostate cancer. British Journal of Cancer, 100(2):426-30.

Kottemann, M.C., and Smogorzewska, A. 2013. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature, 493(7432):356-63.

Krzyzanowski, M., Quackenboss, J.J., and Lebowitz, M.D. 1990. Chronic respiratory effects of indoor formaldehyde exposure. Environmental Research, 52(2):117-25

Kubota, M., Lin, Y.W., Hamahata, K., Sawada, M., Koishi, S., Hirota, H., and Wakazono, Y. 2000. Cancer chemotherapy and somatic cell mutation. Mutation Research, 470: 93-102.

Kumaraswamy, E., and Shiekhattar R. 2007. Activation of BRCA1/BRCA2-associated helicase BACH1 is required for timely progression through S phase. Molecular Cell Biology, 27(19):6733-41.

Kyoizumi, S., Akiyama, M., Hirai, Y., Kusunoki, Y., Tanabe, K., and Umeki, S. 1990. Spontaneous loss and alteration of antigen receptor expression in mature CD4+ T cells. Journal of Experimental Medicine, 171(6):1981-99.

Kyrtopoulos, S.A. 2006. Biomarkers in environmental carcinogenesis research: striving for a new momentum. Toxicology Letters, 162(1):3-15

Ladeira, C., Viegas, S., Carolino, E., Prista, J., Gomes, M.C. and Brito, M. 2011. Genotoxicity biomarkers in occupational exposure to formaldehyde--the case of histopathology laboratories. Mutation Research, 721(1): 15-20.

Ladeira, C., Viegas, S., Carolino, E., Gomes, M.C., and Brito, M. 2013. The influence of genetic polymorphisms in XRCC3 and ADH5 genes on the frequency of genotoxicity biomarkers in workers exposed to formaldehyde. Environmental and Molecular Mutagenesis, 54(3):213-21.

Laffon, B., Teixeira, J.P., Silva, S., Roma-Torres, J., Pérez-Cadahía, B., Méndez, J., Pásaro, E., Mayan, O. 2006. Assessment of occupational genotoxic risk in the production of rubber tyres. Annals of Occupational Hygiene. 50(6):583-92.

Laffon, B., Aguilera, F., Ríos-Vázquez, J., García-Lestón, J., Fuchs, D., Valdiglesias, V., and Pásaro, E. 2013. Endocrine and immunological parameters in individuals involved in Prestige spill cleanup tasks seven years after the exposure. Environment International, 59:103-11.

Lander, B.F., Knudsen, L.E., Gamborg, M.O., Järventaus, H., and Norppa, H. 2000. Chromosome aberrations in pesticide-exposed greenhouse workers. Scandinavian Journal of Work, Environment and Health, 26(5):436-42.

Landi, S. 2000. Mammalian class theta GST and differential susceptibility to carcinogens: a review. Mutation Research, 463(3):247-83.

Lang, I., Bruckner, T., and Triebig, G. 2008. Formaldehyde and chemosensory irritation in humans: a controlled human exposure study. Regulatory Toxicology and Pharmacology, 50(1):23-36.

Lange, S.S., Takata, K., and Wood, R.D. 2011. DNA polymerases and cancer. Nature Reviews Cancer, 11(2):96-110.

Langevin, F., Crossan, G.P., Rosado, I.V., Arends, M.J., and Patel, K.J. 2011. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. Nature, 475(7354):53-8.

Lao, T., Gu, W., and Huang, Q. 2008. A meta-analysis on XRCC1 R399Q and R194W polymorphisms, smoking and bladder cancer risk. Mutagenesis, 23(6): 523-32.

Latt, S.A., and Schreck, R.R. 1980. Sister chromatid exchange analysis. American Journal of Human Genetics, 32(3):297-313.

Lee, X.P., Kumazawa, T., Kondo, K., Sato, K., and Suzuki, O. 1999. Analysis of methanol or formic acid in body fluids by headspace solid-phase microextraction and capillary gas chromatography. J. of Chromatogr. B: Biomedical Sciences and Applications, 734(1): 155–162.

Lee, Y.L., Lin, Y.C., Lee, Y.C., Wang, J.Y., Hsiue, T.R., and Guo, Y.L. 2004. Glutathione S-transferase P1 gene polymorphism and air pollution as interactive risk factors for childhood asthma. Clinical and Experimental Allergy, 34:1707-1713.

Lee, X.P., and Sato, K. 2005. Methanol and Formic Acid. In: Drugs and Poisons in Humans - A Handbook of Practical Analysis. Springer, New York, 123-33 pp.

Lensch,, M.W., Tischkowitz, M., Christianson, T.A., Reifsteck, C.A., Speckhart, S.A., Jakobs, P.M., O'Dwyer, M.E., Olson, S.B., Le Beau, M.M., Hodgson, S.V., Mathew, C.G., Larson, R.A., Bagby, G.C. Jr. 2003. Acquired FANCA dysfunction and cytogenetic instability in adult acute myelogenous leukemia. Blood, 102(1):7-16.

Lin, Y., Dueker, S.R., Jones, A.D., Ebeler, S.E., and Clifford, A.J. 1995. Protocol for collection and HPLC analysis of volatile carbonyl compounds in breath. Clinical Chemistry, 41(7):1028-32.

Lin, D.X., Tang, Y.M., Peng, Q., Lu, S.X., Ambrosone, C.B., Kadlubar, F.F. 1998. Susceptibility to esophageal cancer and genetic polymorphisms in glutathione S-transferases T1, P1, and M1 and cytochrome P450 2E1. Cancer Epidemiology, Biomarkers and Prevention, 7(11):1013-8.

Linos, A., Blair, A., Cantor, K.P., Burmeister, L., VanLier, S., Gibson, R.W., Schuman, L., and Everett, G. 1990. Leukemia and non-Hodgkin's lymphoma among embalmers and funeral directors. Journal of the National Cancer Institute, 82(1):66.

Links, J.M., Kensler, T.W., and Groopman, J.D. 1995. Biomarkers and mechanistic approaches in environmental epidemiology. Annual Review of Public Health, 16:83-103

Liteplo, R.G., and Meek M.E. 2003. Inhaled formaldehyde: exposure estimation, hazard characterization, and exposure-response analysis. Journal of Toxicology and Environmental Health, Part B: Critical Reviews, 6(1):85-114.

Liu, Y., Li, C.M., Lu, Z., Ding, S., Yang, X., Mo, J. 2006. Studies on formation and repair of formaldehyde-damaged DNA by detection of DNA-protein crosslinks and DNA breaks. Frontiers in Bioscience, 11:991-7.

Liu, Y., Jing, H., Wang, J., Zhang, R., Zhang, Y., Zhang, Y., Xu, Q., Yu, X., and Xue, C. 2011. Micronutrients decrease incidence of common infections in type 2 diabetic outpatients. Asian Pacific of Clinical Nutrition, 20(3):375-82.

López-Cima MF, Alvarez-Avellón SM, Pascual T, Fernández-Somoano A, Tardón A. 2012. Genetic polymorphisms in CYP1A1, GSTM1, GSTP1 and GSTT1 metabolic genes and risk of lung cancer in Asturias. BMC Cancer, 412:433.

Lu, K., Ye, W., Gold, A., Ball, L.M., and Swenberg, J.A. 2009. Formation of S-[1-(N2-deoxyguanosinyl)methyl]glutathione between glutathione and DNA induced by formaldehyde. Journal of the American Chemical Society, 131(10):3414-5.

Lucas, D., Ferrara, R., Gonzales, E., Albores, A., Manno, M., and Berthou, F. 2001. Cytochrome CYP2E1 phenotyping and genotyping in the evaluation of health risks from exposure to polluted environments. Toxicology Letters, 124(1-3):71-81.

Luce, D., Gérin, M., Leclerc, A., Morcet, J.F., Brugère, J., Goldberg, M. 1993. Sinonasal cancer and occupational exposure to formaldehyde and other substances. International Journal of Cancer, 53(2):224-31.

Luce, D., Leclerc, A., Bégin, D., Demers, P.A., Gérin, M., Orlowski, E., Kogevinas, M., Belli, S., Bugel, I., Bolm-Audorff, U., Brinton, L.A., Comba, P., Hardell, L., Hayes, R.B., Magnani, C., Merler, E., Preston-Martin, S., Vaughan, T.L., Zheng, W., and Boffetta, P. 2002. Sinonasal cancer and occupational exposures: a pooled analysis of 12 case-control studies. Cancer Causes and Control, 13(2):147-57

Luo, W., Li, H., Zhang, Y., and Ang, C.Y. 2001. Determination of formaldehyde in blood plasma by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography B, Biomedical Sciences and Applications, 753(2):253-7.

Luster, M.I., Ackermann, M.F., Germolec, D.R., and Rosenthal, G.J. 1989. Perturbations of the immune system by xenobiotics. Environmental Health Perspectives, 81:157-62.

Luttrell, W. E., Jederberg, W.W., and Still K. R. 2008. Biomonotoring. In: Toxicology Principles for the Industrial Hygienist. The American Industrial Hygiene Association, Fairfax, US, 352-361 pp.

Lyapina, M., Zhelezova, G., Petrova, E., and Boev, M. 2004. Flow cytometric determination of neutrophil respiratory burst activity in workers exposed to formaldehyde. International Archives of Occupational and Environmental Health, 77(5): 335-340.

Ma, X.D., Cai, G.Q., Zou, W., Huang, Y.H., Zhang, J.R., Wang, D.T., Chen, BL. 2013. BRIP1 variations analysis reveals their relative importance as genetic susceptibility factor for cervical cancer. Biochemical and biophysical Research Communications, 433(2):232-6.

Madison, R.E., Broughton, and A., Thrasher, J.D. 1991. Immunologic biomarkers associated with an acute exposure to exothermic byproducts of a ureaformaldehyde spill. Environmental Health Perspectives, 94: 219-223.

Magaña-Schwencke, N. and Ekert, B. 1978. Biochemical analysis of damage induced in yeast by formaldehyde. II. Induction of cross-links between DNA and protein. Mutation Research, (51):11–19 [abstract].

Manini, P., De Palma, G., and Mutti, A. 2007. Exposure assessment at the workplace: implications of biological variability. Toxicology Letters, 168(3):210-8.

Manuguerra, M., Saletta, F., Karagas, M.R., Berwick, M., Veglia, F., Vineis, P., and Matullo, G. 2006. XRCC3 and XPD/ERCC2 single nucleotide polymorphisms and the risk of cancer: a HuGE review. American journal of epidemiology, 164(4):297-302.

Mapp CE, Fryer AA, De Marzo N, Pozzato V, Padoan M, Boschetto P, Strange RC, Hemmingsen A, and Spiteri MA. 2002. Glutathione S-transferase GSTP1 is a susceptibility gene for occupational asthma induced by isocyanates. Journal Allergy Clinical Immunology, 109(5):867-72.

Marsh, G.M., Youk, A.O., Buchanich, J.M., Erdal, S., and Esmen, N.A. 2007. Work in the metal industry and nasopharyngeal cancer mortality among formaldehyde-exposed workers. Regulatory Toxicology and Pharmacology, 48: 308–319.

Masutani, M., Nakagama, H., and Sugimura, T. 2003. Poly(ADP-ribose) and carcinogenesis. Genes Chromosomes Cancer, 38(4):339-48.

Mateuca, R., Lombaert, N., Aka, P.V., Decordier, I., Kirsch-Volders, M. 2006. Chromosomal changes: induction, detection methods and applicability in human biomonitoring. Biochimie, 88(11):1515-31.

Mateuca, R. and Kirsch-Volders, M. 2007. Chromosomal damage. In: State of validation of biomarkers of carcinogen exposure and early effects and their applicability to molecular epidemiology. Nofer Institute of Occupational medicine. Lodz, Poland, 24–32 pp.

Mateuca, R.A., Roelants, M., Iarmarcovai, G., Aka, P.V., Godderis, L., Tremp, A., Bonassi, S., Fenech, M., Bergé-Lefranc, J.L., Kirsch-Volders, M. 2008. hOGG1 (326), XRCC1(399) and XRCC3(241) polymorphisms influence micronucleus frequencies in human lymphocytes in vivo. Mutagenesis, 23(1):35-41.

Mateuca, R.A., Decordier, I., Kirsch-Volders, M. 2012. Cytogenetic methods in human biomonitoring: principles and uses. Methods in Molecular Biology, 817:305-334.

Mathew, C.G. 2006. Fanconi anaemia genes and susceptibility to cancer. Oncogene. 25(43):5875-84.

Matullo, G., Guarrera, S., Carturan, S., Peluso, M., Malaveille, C., Davico, L., Piazza, A., and Vineis, P. 2001. DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer in a case-control study. International Journal of Cancer, 15;92(4):562-7.

Mautempo, C. 2009. O formaldeído em ambiente laboral: determinação do ácido fórmico em urina de trabalhadores de um fábrica produtora de formaldeído (Formaldehyde in the work environment: determination of formic acid in urine of workers in a factory producing formaldehyde) (MSc Dissertation), Faculade de Farmácia, Universidade do Porto, Porto, 142 pp.

Mautempo, C., Bastos, M. D. L., Carvalho, F., Remião, F., Carmo, H., and Guedes-De-Pinho P. 2010. Determination of formic acid in urine of workers occupationally exposed to formaldehyde. Toxicology Letters, 196S:S74 (P104-023).

Mayan, O., Lacamblez, M., Capela, F., Pinto, L., Freitas, I., Coelho, J. 1995. Formaldeido. Contribuição para o estudo de algumas dos seus efeitos numa população de Técnicos de Laboratório. Revista Saúde e Trabalho, 1(1): 9-51.

Mayeux R. 2004. Biomarkers: potential uses and limitations. NeuroRx 1(2):182-8.

Mazzei, F., Viel, A., and Bignami, M. 2013. Role of MUTYH in human cancer. Mutation Research, 743-744:33-43.

McGregor, D., Bolt, H., Cogliano, V., and Richter-Reichhelm, H.B. 2006. Formaldehyde and glutaraldehyde and nasal cytotoxicity: case study within the context of the 2006 IPCS Human Framework for the Analysis of a cancer mode of action for humans. Critical Reviews of Toxicology, 36(10): 821-835.

McGwin, G., Lienert, J., and Kennedy, J.I. 2010. Formaldehyde exposure and asthma in children:a systematic review. Environmental Health Perspectives, 118(3):313-7.

Merk, O. and Speit, G. 1998. Significance of formaldehyde-induced DNA–protein crosslinks for mutagenesis. Environmental and Molecular Mutagenesis, 32(3):260-268.

Meyers, A.R., Pinkerton, L.E., and Hein, M.J. 2013. Cohort mortality study of garment industry workers exposed to formaldehyde: update and internal comparisons. American Journal of Industrial Medicine, 56(9):1027-39.

Migliore, L., Ventura, L., Barale, R., Loprieno, N., Castellino, S., and Pulci, R.1989. Micronuclei and nuclear anomalies induced in the gastro-intestinal epithelium of rats treated with formaldehyde. Mutagenesis, 4(5):327-34.

Mo, Z., Gao, Y., Cao, Y., Gao, F., and Jian, L. 2009. An updating meta-analysis of the GSTM1, GSTT1, and GSTP1 polymorphisms and prostate cancer: a HuGE review. Prostate, 69(6):662-88.

Mocellin, S., Verdi, D., and Nitti, D. 2009. DNA repair gene polymorphisms and risk of cutaneous melanoma: a systematic review and meta-analysis. Carcinogenesis, 30(10):1735-43.

Moldovan, G.L., and D'Andrea, A.D. 2009. How the fanconi anemia pathway guards the genome. Annual Review of Genetics, 43:223-49.

Montaldo, E., Del Zotto, G., Della Chiesa, M., Mingari, M.C., Moretta, A., De Maria, A., Moretta, L. 2013. Human NK cell receptors/markers: a tool to analyze NK cell development, subsets and function. Cytometry A, 83(8):702-13.

Monticello, T.M., Morgan, K.T., Everitt, J.I., and Popp, J.A. 1989. Effects of formaldehyde gas on the respiratory tract of rhesus monkeys. Pathology and cell proliferation. American Journal Pathology, 134(3):515-27.

Monticello, T.M., Swenberg, J.A., Gross, E.A., Leininger, J.R., Kimbell, J.S., Seilkop, S., Starr, T.B., Gibson, J.E., and Morgan KT. 1996. Correlation of regional and nonlinear formaldehyde-induced nasal cancer with proliferating populations of cells. Cancer Research, 56(5):1012-22.

Monticello, T.M., and Morgan, K.T. 1997. Chemically-induced nasal carcinogenesis and epithelial cell proliferation: a brief review. Mutation Research, 380 (1-2):33-41.

Moser, B., Bodrogi, F., Eibl, G., Lechner, M., Rieder, J., and Lirk, P. 2005. Mass spectrometric profile of exhaled breath--field study by PTR-MS. Respiratory Physiology and Neurobiology, 145(2-3):295-300.

Moses, R.E. 2001. DNA damage processing defects and disease. Annual Review Genomics and Human Genetics, 2:41-68.

Moszczyński P, Zabiński Z, Moszczyński P Jr, Rutowski J, Słowiński S, Tabarowski Z. 2001. Immunological findings in cigarette smokers. Toxicology Letters, 118(3):121-7.

Murgia, E., Ballardin, M., Bonassi, S., Rossi, A.M., and Barale, R. 2008. Validation of micronuclei frequency in peripheral blood lymphocytes as early cancer risk biomarker in a nested case-control study. Mutation Research, 639 (1-2):27-34.

Musak, L., Smerhovsky, Z., Halasova, E., Osina, O., Letkova, L., Vodickova, L., Polakova, V., Buchancova, J., Hemminki, K., Vodicka, P. 2013. Chromosomal damage among medical staff occupationally exposed to volatile anesthetics, antineoplastic drugs, and formaldehyde. Scandinavian Journal of Work, Environment and Health, 39(6):618-30.

Natarajan, A.T., Tucker, J.D., and Sasaki, M.S. 1994. Monitoring cytogenetic damage in vivo. In: SCOPE 52 Methods to Assess DNA Damage and Repair: Interspecies Comparisons. John Wiley and Sons, 95-116 pp. http://dge.stanford.edu/SCOPE/SCOPE_52/SCOPE_52_2.04_Natarajan_95-116.pdf

NTP (National Toxicology Program). 2011. Report on Carcinogens, Twelfth Edition-Formaldehyde. Research Triangle Park, NC: US Department of Health and Human Services, Public Health Service, National Toxicology Program, 195-205 pp.

Neri, M., Bonassi, S., Knudsen, L.E., Sram, R.J., Holland, N., Ugolini, D., and Merlo, D.F. 2006. Children's exposure to environmental pollutants and biomarkers of genetic damage I. Overview and critical issues. Mutation Research, 612(1):1-13.

Nielsen, G.D., and Wolkoff, P. 2010. Cancer effects of formaldehyde: a proposal for an indoor air guideline value. Archives of Toxicology, 84(6):423-46.

NIOSH (National Institute for Occupational Safety and Health). 1994. Formaldehyde: Method 3500, Issue 2 in NIOSH Manual of Analytical Method, 4th Edition. U.S. Department of Health and Human Services, Cincinnati, Ohio.

Nedelcheva, V., Persson, I., and Ingelman-Sundberg, M. 1996. Genetic polymorphism of human cytochrome P450 2E1. Methods in Enzymology, 272: 216-225.

Nersesyan, A., Kundi, M., Atefie, K., Schulte-Hermann, R., and Knasmüller, S. 2006. Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. Cancer Epidemiology, Biomarkers and Prevention, 15(10):1835-40.

Nersesyan, A., Muradyan, R., Kundi, M., and Knasmueller, S. 2011. Impact of smoking on the frequencies of micronuclei and other nuclear abnormalities in exfoliated oral cells: a comparative study with different cigarette types. Mutagenesis, 26(2):295-301.

Neuss, S., and Speit, G. 2008. Further characterization of the genotoxicity of formaldehyde in vitro by the sister chromatid exchange test and co-cultivation experiments. Mutagenesis, 23(5):355-7.

Norppa, H., Bonassi, S., Hansteen, I.L., Hagmar, L., Strömberg, U., Rössner, P., Boffetta, P., Lindholm, C., Gundy, S., Lazutka, J., Cebulska-Wasilewska, A., Fabiánová, E., Srám, R.J., Knudsen, L.E., Barale, R., and Fucic, A. 2006. Chromosomal aberrations and SCEs as biomarkers of cancer risk. Mutation Research, 600(1-2):37-45.

Obe, G., Pfeiffer, P., Savage, J.R., Johannes, C., Goedecke, W., Jeppesen, P., Natarajan, A.T., Martínez-López, W., Folle, G.A., and Drets, M.E. 2002. Chromosomal aberrations: formation, identification and distribution. Mutation Research, 504(1-2):17-36.

Odeigah, P.G. 1997. Sperm head abnormalities and dominant lethal effects of formaldehyde in albino rats. Mutation Research, 389(2-3):141-8.

Oh, E., Lee, E., Im, H., Kang, H.-S., Jung, W.-W., Won, N.H., Kim, E.-M., and Sul, D. 2005. Evaluation of immuno- and reproductive toxicities and association between immunotoxicological and genotoxicological parameters in waste incineration workers. Toxicology, 210: 65-80.

Ohmichi K., Matsuno Y., Miyaso H., Yamamoto H., Toriuchi M., Shimane M., Mori C. 2006. Pilot study of a dissection table for gross anatomy laboratory equipped with a photocatalytic device that decomposes formaldehyde. Journal Occupational of Health, 49(6): 499-503.

Olsen, J.H., and Asnaes, S. 1986. Formaldehyde and the risk of squamous cell carcinoma of the sinonasal cavities. British Journal of Industrial Medicine, 43(11):769-74

Olsen, N.J., Visell, S.M., Fan, J., and Kovacs, W.J. 1998. Androgens accelerate thymocyte apoptosis. Endocrinology, 139(2):748-52.

Oostra, A.B., Nieuwint, A.W., Joenje, H., and de Winter, J.P. 2012. Diagnosis of fanconi anemia: chromosomal breakage analysis. Anemia, 2012:238731.

Oota, H., Pakstis, A.J., Bonne-Tamir, B., Goldman, D., Grigorenko, E., Kajuna, S.L., Karoma, N.J., Kungulilo, S., Lu, R.B., Odunsi, K., Okonofua, F., Zhukova, O.V., Kidd, J.R., and Kidd, K.K. 2004. The evolution and population genetics of the ALDH2 locus: random genetic drift, selection, and low levels of recombination. Annals of Human Genetics, 68 (Pt 2):93-109.

Orsière, T., Sari-Minodier, I., Iarmarcovai, G., and Botta, A. 2006. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. Mutation Research, 605(1-2): 30-41.

OSHA (Occupational Safety and Health Standards). 2011. OSHA fact sheet-formaldehyde. OSHA, 2pp. https://www.osha.gov/OshDoc/data_General_Facts/

formaldehyde-factsheet.pdf. (consulted on 17th of November, 2013).

Out, A.A., Wasielewski, M., Huijts, P.E., van Minderhout, I.J., Houwing-Duistermaat, J.J., Tops, C.M., Nielsen, M., Seynaeve, C., Wijnen, J.T., Breuning, M.H., van Asperen, C.J.,

Schutte, M., Hes, F.J., and Devilee, P. MUTYH gene variants and breast cancer in a Dutch case–control study. Breast Cancer Research Treatment, 134(1):219-27.

Øvrebø, S., Haugen, A., and Skaug, V. 2002. Biotransformation of formaldehyde in cultured human bronchus. Environmental Research, 89(1):38-42.

Pala, M., Ugolini, D., Ceppi, M., Rizzo, F., Maiorana, L., Bolognesi, C., Schilirò, T., Gilli, G., Bigatti, P., Bono, R., and Vecchio, D. 2008. Occupational exposure to formaldehyde and biological monitoring of Research Institute workers. Cancer Detection and Prevention, 32(2):121-6.

Palitti, F. 1998. Mechanisms of the origin of chromosomal aberrations. Mutation Research, 404(1-2):133-137.

Park, D.J., Lesueur, F., Nguyen-Dumont, T., Pertesi, M., Odefrey, F., Hammet, F., Neuhausen, S.L., John, E.M., Andrulis, I.L., Terry, M.B., Daly, M., Buys, S., Le Calvez-Kelm, F., Lonie, A., Pope, B.J., Tsimiklis, H., Voegele, C., Hilbers, F.M., Hoogerbrugge, N., Barroso, A., Osorio, A; Breast Cancer Family Registry; Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer, Giles, G.G., Devilee, P., Benitez, J., Hopper, J.L., Tavtigian, S.V., Goldgar, D.E., and Southey, M.C. 2012. Rare mutations in XRCC2 increase the risk of breast cancer. American Journal of Human Genetics, 90(4):734-9.

Partanen, T., Kauppinen, T., Luukkonen, R., Hakulinen, T., Pukkala, E. 1993. Malignant lymphomas and leukemias, and exposures in the wood industry: an industry-based case-referent study. International archives of occupational and environmental health 64(8):593-6.

Pastinen, T., Sladek, R., Gurd, S., Sammak, A., Ge, B., Lepage, P., Lavergne, K., Villeneuve, A., Gaudin, T., Brändström, H., Beck, A., Verner, A., Kingsley, J., Harmsen, E., Labuda, D., Morgan, K., Vohl, M.C., Naumova, A.K., Sinnett, D., and Hudson, T.J. 2004. A survey of genetic and epigenetic variation affecting human gene expression. Physiological Genomics, 16(2):184-93.

Pavanello, S., and Clonfero, E. 2000. Biological indicators of genotoxic risk and metabolic polymorphisms. Mutation Research, 463(3):285-308.

Pazdrak, K., Górski, P., Krakowiak, A., and Ruta, U. 1993. Changes in nasal lavage fluid due to formaldehyde inhalation. International Archives of Occupational and Environmental Health 64(7):515-9 [abstract].

Paz-y-Miño, C., Dávalos, M.V., Sánchez, M.E., Arévalo, M., and Leone, P.E. 2002. Should gaps be included in chromosomal aberration analysis? Evidence based on the comet assay. Mutation Research, 516(1-2):57-61.

Pawliszyn J.1999. Applications of Solid-phase Microextraction. Royal Society of Chemistry- Chromatography monographs, Cambridge, UK, 654 pp.

Peck, E.C., and Eaton, and D.L.2010. Genetic polymorphisms and Susceptibility to Pesticides. In: Hayes' Handbook of Pesticides Toxicology R. Krieger ed. (Elsevier Inc.), 525-529 pp.

Pennington, K.P., and Swisher, E.M. 2012. Hereditary ovarian cancer: beyond the usual suspects. Gynecologic Oncology. 124(2):347-53.

Perera, F. P. 1997. Environment and cancer: who are susceptible? Science, 278:1068-1073

Pérez, L.O., Crivaro, A., Barbisan, G., Poleri, L., and Golijow, C.D. 2013. XRCC2 R188H (rs3218536), XRCC3 T241M (rs861539) and R243H (rs77381814) single nucleotide polymorphisms in cervical cancer risk. Pathology Oncology Research,19(3):553-8.

Perry, P., and Wolff, S. 1974. New Giemsa method for the differential staining of sister chromatids. Nature, 251: 156-158.

Petrozzi, L., Lucetti, C., Scarpato, R., Gambaccini, G., Trippi, F., Bernardini, S., Del Dotto, P., Migliore, L., and Bonuccelli, U. 2002. Cytogenetic alterations in lymphocytes of Alzheimer's disease and Parkinson's disease patients. Neurology Sciences, 23(Suppl 2):S97-8.

Pfeiffer, P., Goedecke, W., Obe, G. 2000. Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. Mutagenesis, 15(4):289-302.

Pierce, J.S., Abelmann, A., Spicer, L.J., Adams, R.E., Glynn, M.E., Neier, K., Finley, B.L., and Gaffney, S.H. 2011. Characterization of formaldehyde exposure resulting from the use of four professional hair straightening products. Journal of Occupational and Environmental Hygiene. 8(11):686-99.

Picelli, S., Zajac, P., Zhou, X.L., Edler, D., Lenander, C., Dalén, J., Hjern, F., Lundqvist, N., Lindforss, U., Påhlman, L., Smedh, K., Törnqvist, A., Holm, J., Janson, M., Andersson, M., Ekelund, S., Olsson, L., Lundeberg, J., and Lindblom, A. 2010. Common variants in human CRC genes as low-risk alleles. European Journal of Cancer, 46(6):1041-8.

Pierdominici, M., Maselli, A., Colasanti, T., Giammarioli, A.M., Delunardo, F., Vacirca, D., Sanchez, M., Giovannetti, A., Malorni, W., and Ortona, E. 2010. Estrogen receptor profiles in human peripheral blood lymphocytes. Immunology Letters, 132(1-2):79-85.

Pilidis, G.A., Karakitsios, S.P., Kassomenos, P.A., Kazos, E.A., and Stalikas, C.D. 2008. Measurements of benzene and formaldehyde in a medium sized urban environment. Indoor/outdoor health risk implications on special population groups. Environmental Monitoring Assesment, 150(1-4):285-94.

Pinkerton, L.E., Hein, M.J., Stayner, L.T. 2004. Mortality among a cohort of garment workers exposed to formaldehyde: an update. Occupational and Environmental Medicine, 61(3): 193-200.

Piperakis, S.M. 2009. Comet assay: a brief history. Cell Biology Toxicology, 25(1):1-3.

Proia NK, Paszkiewicz GM, Nasca MA, Franke GE, Pauly JL. 2006. Smoking and smokeless tobacco-associated human buccal cell mutations and their association with oral cancer--a review. Cancer Epidemiology, Biomarkers and Prevention, 15(6):1061-77.

Qian, B., Zhang, H., Zhang, L., Zhou, X., Yu, H., and Chen, K. 2011. Association of genetic polymorphisms in DNA repair pathway genes with non-small cell lung cancer risk. Lung Cancer, 73(2):138-46.

Qiu, H., and Wang, Y. 2009. Exploring DNA-binding proteins with in vivo chemical cross-linking and mass spectrometry. Journal Proteome Research, 8: 1983-1991.

Rager, J.E., Smeester, L., Jaspers, I., Sexton, K.G., and Fry, R.C. 2011. Epigenetic changes induced by air toxics: formaldehyde exposure alters miRNA expression profiles in human lung cells. Environmental Health Perspectives, 119(4):494-500.

Rager, J.E., Moeller, B.C., Doyle-Eisele, M., Kracko, D., Swenberg, J.A., and Fry, R.C. 2013. Formaldehyde and epigenetic alterations: microRNA changes in the nasal epithelium of nonhuman primates. Environmental Health Perspectives, 121(3):339-44.

Raimondi S., Paracchini V., Autrup H., Barros-Dios J.M., Benhamou S., Boffetta P., Cote, M.L., Dialyna, I.A., Dolzan, V., Filibert,i R., Garte, S., Hirvonen, A., Husgafvel-Pursiainen, K., Imyanitov, E.N., Kalina, I., Kang, D., Kiyohara, C., Kohno, T., Kremers, P., Lan, Q., London, S., Povey, A.C., Rannug, A., Reszka, E., Risch, A., Romkes, M., Schneider, J., Seow, A., Shields, P.G., Sobti, R.C., Sørensen, M., Spinola, M., Spitz, M.R., Strange, R.C., Stücker, I., Sugimura, H., To-Figueras, J., Tokudome, S., Yang, P., Yuan, J.M., Warholm, M., and Taioli, E. 2006. Meta- and pooled analysis of GSTT1 and lung cancer: a HuGE-GSEC review. American Journal of Epidemiology, 164(11): 1027-42.

Ramalho, A.T., Curado, M.P., and Natarajan, A.T. 1995. Lifespan of human lymphocytes estimated during a six year cytogenetic follow-up of individuals accidentally exposed in the 1987 radiological accident in Brazil. Mutation Research, 331(1): 47-54.

Raucy, J.L., Curley, G., and Carpenter, S.P. 1995. Use of lymphocytes for assessing ethanol-mediated alterations in the expression of hepatic cytochrome P4502E1. Alcohol, Clinical and Experimental Research, 19(6):1369-75.

Raucy, J.L., Schultz, E.D., Wester, M.R., Arora, S., Johnston, D.E., Omdahl, J.L., and Carpenter, S.P. 1997. Human lymphocyte cytochrome P450 2E1, a putative marker for alcohol-mediated changes in hepatic chlorzoxazone activity. Drug Metabolism and Disposition, 25(12):1429-35.

Richardson, C. 2005. RAD51, genomic stability, and tumorigenesis. Cancer Letters, 218(2):127-39.

Ridpath JR, Nakamura A, Tano K, Luke AM, Sonoda E, Arakawa H, Buerstedde JM, Gillespie DA, Sale JE, Yamazoe M, Bishop DK, Takata M, Takeda S, Watanabe M, Swenberg JA, Nakamura J. 2007. Cells deficient in the FANC/BRCA pathway are hypersensitive to plasma levels of formaldehyde. Cancer Research, 67(23):11117-22.

Ricks-Santi, L.J., Sucheston, L.E., Yang, Y., Freudenheim, J.L., Isaacs, C.J., Schwartz, M.D., Dumitrescu, R.G., Marian, C., Nie, J., Vito, D., Edge, S.B., and Shields, P.G. Association of Rad51 polymorphism with DNA repair in BRCA1 mutation carriers and sporadic breast cancer risk. BMC Cancer, 11:278.

Rodríguez-Reyes, R., and Morales-Ramírez, P. 2003. Sister chromatid exchange induction and the course of DNA duplication, two mechanisms of sister chromatid exchange induction by ENU and the role of BrdU. Mutagenesis, 18(1):65-72.

Romanowicz, H., Smolarz, B., Baszczyński, J., Zadrożny, M., and Kulig A. 2010. Genetics polymorphism in DNA repair genes by base excision repair pathway (XRCC1) and homologous recombination (XRCC2 and RAD51) and the risk of breast carcinoma in the Polish population. Polish Journal Pathology, 61(4):206-12.

Ronen, A., and Glickman, B.W. 2001. Human DNA repair genes. Environmental and Molecular Mutagenesis, 37(3):241-83.

Rosado, I. V., Langevin, F., Crossan, G. P., Takata, M. and Patel, K. J. 2011. Formaldehyde catabolism is essential in cells deficient for the Fanconi anemia DNA-repair pathway. Nature Structural and Molecular Biology, 18: 1432–1434.

Rosin, M.P. 1992. The use of the micronucleus test on exfoliated cells to identify anti-clastogenic action in humans: a biological marker for the efficacy of chemopreventive agents. Mutation Research, 267(2):265-76.

Rossi, A.M., Hansteen, I.L., Skjelbred, C.F., Ballardin, M., Maggini, V., Murgia, E., Tomei, A., Viarengo, P., Knudsen, L.E., Barale, R., Norppa, H., and Bonassi, S. 2009. Association between frequency of chromosomal aberrations and cancer risk is not influenced by genetic polymorphisms in GSTM1 and GSTT1. Environmental Health Perspectives, 117(2):203-8.

Rossner, P., Boffetta, P., Ceppi, M., Bonassi, S., Smerhovsky, Z., Landa, K., Juzova, D., Srám R.J. 2005. Chromosomal aberrations in lymphocytes of healthy subjects and risk of cancer. Environmental Health Perspectives, 113(5):517-20.

Roush, G.C., Walrath, J., Stayner, L.T., Kaplan, S.A., Flannery, J.T., Blair, A.1987. Nasopharyngeal cancer, sinonasal cancer, and occupations related to formaldehyde: a case-control study. Journal of the National Cancer Institute, 79(6):1221-4 [abstract].

Rueff, J., Gaspar, J., and Kranendonk, M. 2002. DNA polymorphisms as modulators of genotoxicity and cancer. Biological Chemistry, 383(6):923-32.

Rumchev, K.B., Spickett, J.T., Bulsara, M.K., Phillips, M.R., and Stick S.M. 2002. Domestic exposure to formaldehyde significantly increases the risk of asthma in young children. European Respiratory Journal, 20:403–408.

Russo, A., Francelin, P.R., Galbiatti, A.L., Raposo, L.S., Maníglia, J.V., Pavarino, E.C., and Goloni-Bertollo, E.M. 2013. Association between GSTP1, GSTM1 and GSTT1 polymorphisms involved in xenobiotic metabolism and head and neck cancer development. Molecular Biology Report, 40(7):4181-8.

Ruwali, M., Singh, M., Pant, M.C., and Parmar, D. 2011. Polymorphism in glutathione S-transferases: susceptibility and treatment outcome for head and neck cancer. Xenobiotica, 41(12):1122-30.

Saito Y, Nishio K, Yoshida Y, and Niki E. 2005. Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species. Toxicology, 210: 235-245.

Salama, S.A., Serrana, M., and Au, W.W. 1999. Biomonitoring using accessible human cells for exposure and health risk assessment. Mutation Research, 436(1):99-112.

Salthammer, T., Mentese, S., and Marutzky, R. 2010. Formaldehyde in the indoor environment. Chemical Reviews, 110(4):2536-72.

Santagostino, A., Garbaccio, G., Pistorio, A., Bolis, V., Camisasca, G., Pagliaro, P., and Girotto, M. 1999. An Italian national multicenter study for the definition of reference ranges for normal values of peripheral blood lymphocyte subsets in healthy adults. Haematologica, 84(6):499-504.

Santonocito, C., Scapaticci, M., Penitente, R., Paradisi, A., Capizzi, R., Lanza-Silveri, S., Ficarra, S., Landi, F., Zuppi, C., and Capoluongo, E. 2012. Polymorphisms in base excision DNA repair genes and association with melanoma risk in a pilot study on Central-South Italian population. Clinica Chimica Acta. 413(19-20):1519-24.

Santos, L.S., Branco, S.C., Silva, S.N., Azevedo, A.P., Gil, O.M., Manita, I., Ferreira, T.C.,

Limbert, E., Rueff, J., and Gaspar, J.F. 2012. Polymorphisms in base excision repair genes and thyroid cancer risk. Oncology Reports, 28(5):1859-68.

Saule, P., Trauet, J., Dutriez, V., Lekeux, V., Dessaint, J.P., and Labalette, M. 2006. Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment. Mechanisms of Ageing and Development, 127(3):274-81.

Savage, J.R.K. 2004. On the nature of visible chromosomal gaps and breaks. Cytogenetics Genome Research, 104(1-4):46-55.

Savage, J.R.K. 1975. Classification and relationships of induced chromosomal structural changes. Journal Medicine Genetics, 13(2):103–122.

Schmid, E., Göggelmann, W., and Bauchinger, M. 1986. Formaldehyde-induced cytotoxic, genotoxic and mutagenic response in human lymphocytes and Salmonella typhimurium. Mutagenesis, 1(6):427-31.

Schmid K., Schaller K.H., Angerer J, Lehnert G. 1994. The Importance of formic acid Excretion in the urine for environmental and occupational medicine questions. Zentralbl Hyg Umweltmed, 196(2): 139-152 [abstract].

Schmid, O., and Speit, G. 2007. Genotoxic effects induced by formaldehyde in human blood and implications for the interpretation of biomonitoring studies. Mutagenesis, 22(1): 69-74.

Schlacher K, Wu H, Jasin M. 2012. A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. Cancer Cell, 22(1):106-16.

Schroeder, T.M., and German, J. 1974. Bloom's syndrome and Fanconi's anemia: demonstration of two distinctive patterns of chromosome disruption and rearrangement. Humangenetik, 25(4):299-306.

Schulz, T.F. 2009. Cancer and viral infections in immunocompromised individuals. International Journal Cancer, 15;125(8):1755-63.

SCOEL (Scientific Committee on Occupational Exposure Limit Values). 2008. Recommendation from the Scientific Committee on Occupational Exposure Limits for Formaldehyde. SCOEL/SUM/125, European Commission.

http://ec.europa.eu/social/keyDocuments.jsp?policyArea=&type=0&country=0&year=0&advSearchKey=formaldehyde&mode=advancedSubmit&langId=en (consulted on 17th of November, 2013).

Sellakumar, A.R., Snyder, C.A., Solomon, J.J. and Albert, R.E. 1985. Carcinogenicity of formaldehyde and hydrogen chloride in rats. Toxicology applied Pharmacology, 81, 401–406

Shaham, J., Bomstein, Y., Meltzer, A., and Ribak, J. 1997. DNA-protein crosslinks and sister chromatid exchanges as biomarkers of exposure to formaldehyde. International Journal of Occupational and Environmental Health, 3: 95-104.

Shaham, J., Gurvich, R., and Kaufman, Z. 2002. Sister chromatid exchange in pathology staff occupationally exposed to formaldehyde. Mutation Research, 15:115-123.

Shaham, J., Bomstein, Y., Gurvich, R., Rashkovsky, M., and Kaufman, Z. 2003. DNA–protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde. Occupational and Environmental Medicine, 60:403–409

Shahriary, G.M., Galehdari, H., Jalali, A., Zanganeh, F., Alavi, S.M., and Aghanoori, M.R. 2012. CYP2E1*5B, CYP2E1*6, CYP2E1*7B, CYP2E1*2, and CYP2E1*3 Allele Frequencies in Iranian Populations. Asian Pacific Journal of Cancer Prevention, 13(12):6505-10.

Sigurdson, A.J., Hauptmann, M., Chatterjee, N., Alexander, B.H., Doody, M.M., Rutter, J.L., and Struewing JP. 2004. Kin-cohort estimates for familial breast cancer risk in relation to variants in DNA base excision repair, BRCA1 interacting and growth factor genes. BMC Cancer, 12;4:9.

Silva, Mdo C., Gaspar, J., Duarte Silva, I., Faber, A., and Rueff, J. 2004. GSTM1, GSTT1, and GSTP1 genotypes and the genotoxicity of hydroquinone in human lymphocytes. Environmental and Molecular Mutagenesis, 43(4):258-64.

Silva, S.N., Tomar, M., Paulo, C., Gomes, B.C., Azevedo, A.P., Teixeira, V., Pina, J.E., Rueff, J., and Gaspar, J.F. 2010. Breast cancer risk and common single nucleotide polymorphisms in homologous recombination DNA repair pathway genes XRCC2, XRCC3, NBS1 and RAD51. Cancer Epidemiology, 34(1): 85-92.

Singh, N., McCoy, M., Tice, R., and Schneider, E. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research, 175: 184-191.

Skjelbred, C.F., Svendsen, M., Haugan, V., Eek, A.K., Clausen, K.O., Kure, E.H., Tuimala, J.T., Svendsen, M.V., Norppa, H., and Hansteen I.L. 2011. Influence of GSTM1, GSTT1, GSTP1, NAT1, NAT2, EPHX1, MTR and MTHFR polymorphism on chromosomal aberration frequencies in human lymphocytes. Carcinogenesis, 32(3):399-405.

Smerhovsky Z, Landa K, Rössner P, Brabec M, Zudova Z, Hola N, Pokorna Z, Mareckova J, Hurychova D. 2001. Risk of cancer in an occupationally exposed cohort with increased level of chromosomal aberrations. Environmental Health Perspectives, 109(1):41-5.

Smerhovsky, Z., Landa, K., Rössner, P., Juzova, D., Brabec, M., Zudova, Z., Hola, N., Zarska, H., and Nevsimalova, E. 2002. Increased risk of cancer in radon-exposed miners with elevated frequency of chromosomal aberrations. Mutation Research, 514(1-2):165-76.

Soffritti, M., Maltoni, C., Maffei, F., and Biagi, R. 1989. Formaldehyde: an experimental multipotential carcinogen. Toxicology Industrial Health, 5(5):699-730.

Soffritti, M., Belpoggi, F., Lambertin, L., Lauriola, M., Padovani, M., and Maltoni, C.2002. Results of long-term experimental studies on the carcinogenicity of formaldehyde and acetaldehyde in rats. Annals of New York Academy of Sciences, 982:87-105.

Speit, G. 2006. The implausibility of systemic genotoxic effects measured by the comet assay in rats exposed to formaldehyde. Journal of Proteome Research, 5(10):2523-4

Speit, G., Schmid, O., Neuss, S., and Schütz, P. 2008. Genotoxic effects of formaldehyde in the human lung cell line A549 and in primary human nasal epithelial cells. Environmental Molecular Mutagenesis, 49(4):300-7.

Speit, G., Kühner, S., Linsenmeyer, R., and Schütz, P. 2011. Does formaldehyde induce aneuploidy? Mutagenesis, 26(6):805-11.

Spivack, S.D., Hurteau, G.J., Jain, R., Kumar, S.V., Aldous, K.M., Gierthy, J.F., and Kaminsky, L.S. 2004. Gene-environment interaction signatures by quantitative mRNA profiling in exfoliated buccal mucosal cells. Cancer Research, 64(18):6805-13.

Stern, M.C., Johnson, L.R., Bell, D.A., and Taylor, J.A. 2002. XPD codon 751 polymorphism, metabolism genes, smoking, and bladder cancer risk. Cancer epidemiology, biomarkers and prevention, 11:1004–1011.

Stephens, E.A., Taylor, J.A, Kaplan, N., Yang, C.H., Hsieh, L.L., Lucier, G.W,, Bell, DA.1994.Ethnic variation in the CYP2E1 gene, polymorphism analysis of 695 African-Americans, European Americans and Taiwanese. Pharmacogenetics, 4: 185-192.

Strange, R.C., Spiteri, M.A., Ramachandran, S., and Fryer, A.A. 2001. Glutathione-S-transferase family of enzymes. Mutation Research, 482(1-2):21-6.

Sørensen, M., Autrup, H., Tjønneland, A., Overvad, K., and Raaschou-Nielsen, O. 2004. Glutathione S-transferase T1 null-genotype is associated with an increased risk of lung cancer. International journal of cancer, 110(2):219-24.

Sørensen, M., Raaschou-Nielsen, O., Brasch-Andersen, C., Tjønneland, A., Overvad, K., and Autrup, H. 2007. Interactions between GSTM1, GSTT1 and GSTP1 polymorphisms and smoking and intake of fruit and vegetables in relation to lung cancer. Lung Cancer, 55(2):137-44.

Su, X., and Huang, J. 2011. The Fanconi anemia pathway and DNA interstrand cross-link repair. Protein and Cell, 2(9):704-11.

Sul, D., Lee, D., Im, H., Oh, E., Kim, J., and Lee, E. 2002. Single strand DNA breaks in T- and B-lymphocytes and granulocytes in workers exposed to benzene. Toxicology Letters, 134(1-3):87-95.

Sul, D., Oh, E., Im, H., Yang, M., Kim, C.W., and Lee, E. 2003. DNA damage in T- and B-lymphocytes and granulocytes in emission inspection and incineration workers exposed to polycyclic aromatic hydrocarbons. Mutation Research, 538(1-2):109-19.

Sul, D., Kim, H., Oh, E., Phark, S., Cho, E., Choi, S., Kang, H.S., Kim, E.M., Hwang, K.W., and Jung, W.W. 2007. Gene expression profiling in lung tissues from rats exposed to formaldehyde. Archives of Toxicology, 81(8): 589-97.

Suruda, A., Schulte, P., Boeniger, M., Hayes, R.B., Livingston, G.K. and Steenland, K. 1993. Cytogenetic effects of formaldehyde exposure in students of mortuary science. Cancer Epidemiology, Biomarkers and Prevention, 2: 453-460.

Suspiro, A., and Prista, J. 2011. Biomarkers of occupational exposure do anticancer agents: a minireview. Toxicology Letters, 207(1):42-52.

Takahashi, S., Tsuji, K., Fujii, K., Okazaki, F., Takigawa, T., Ohtsuka, A., Iwatsuki, K. 2007. Prospective study of clinical symptoms and skin test reactions in medical students exposed to formaldehyde gas. Journal of Dermatology, 34(5):283–289.

Takeuchi, A., Takigawa, T., Abe, M., Kawai, T., Endo, Y., Yasugi, T., Endo, G., and Ogino, K. 2007. Determination of formaldehyde in urine by headspace gas chromatography. Bulletin of Environmental Contamination and Toxicology, 79(1):1-4.

Talalay, P., Prochaska, H.J., and Spencer, S.R. 1990. Regulation of enzymes that detoxify the electrophilic forms of chemical carcinogens. Princess Takamatsu Symposium, 21:177-87.

Tambini, C.E., Spink, K.G., Ross, C.J., Hill, M.A., and Thacker, J. 2010. The importance of XRCC2 in RAD51-related DNA damage repair. DNA Repair (Amst), 9(5):517-25.

Tang, X., Bai, Y., Duong, A., Smith, M.T., Li, L., Zhang, L. 2009. Formaldehyde in China: Production, consumption, exposure levels, and health effects. Environmental International, 35(8):1210-24.

Taooka, Y., Takeichi, N., Noso, Y., Kawano, N., Apsalikov, K.N., and Hoshi, M. 2006. Increased T-cell receptor mutation frequency in radiation-exposed residents living near the Semipalatinsk nuclear test site. Journa of Radiation Research, 47 (Suppl A):A179-81.

Teixeira, J.P., Gaspar, J., Martinho, G., Silva, S., Rodrigues, S., Mayan, O., Martin, E., Farmer, P.B., Rueff, J. 2002. Aromatic DNA adduct levels in coke oven workers: correlation with polymorphisms in genes GSTP1, GSTM1, GSTT1 and CYP1A1. Mutation Research, 517(1-2):147-55.

Teixeira J., Gaspar J., Silva S., Torres J., Silva S., Azevedo M., Neves P., Laffon B., Mendez J., Goncalves C., Mayan O., Farmer P., Rueff J. 2004. Occupational exposure to styrene: modulation of cytogenetic damage and levels of urinary metabolites of styrene by polymorphisms in genes CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1. Toxicology, 195: 231-42.

Teng, S., Beard, K., Pourahmad, J., Moridani, M., Easson, E., Poon, R., and O'Brian PJ. 2001. The formaldehyde metabolic detoxification enzyme systems and molecular cytotoxic mechanism in isolated rat hepatocytes. Chemico-Biological Interactions, 130-132: 285-296.

Tischkowitz, M.D., Morgan, N.V., Grimwade, D., Eddy, C., Ball, S., Vorechovsky, I., Langabeer, S., Stöger, R., Hodgson, S.V., and Mathew, C.G. 2004. Deletion and reduced expression of the Fanconi anemia FANCA gene in sporadic acute myeloid leukemia. Leukemia, 18(3):420-5.

Tischkowitz, M., Easton, D.F., Ball, J., Hodgson, S.V., and Mathew, C.G. 2008. Cancer incidence in relatives of British Fanconi Anaemia patients. BMC Cancer, 8:257.

Titenko-Holland, N., Levine, A.J., Smith, M.T., Quintana, P.J., Boeniger, M., Hayes, R., Suruda, A. and Schulte, P. 1996. Quantification of epithelial cell micronuclei by fluorescence in situ hybridization (FISH) in mortuary science students exposed to formaldehyde. Mutation Research, 371: 237-48.

Thacker, J., and Zdzienicka, M.Z. 2003. The mammalian XRCC genes: their roles in DNA repair and genetic stability. DNA Repair (Amst), 2(6):655-72.

Thacker, J. 2005. The RAD51 gene family, genetic instability and cancer. Cancer Letters, 219(2):125-35.

Thomas, P., Hecker, J., Faunt, J., and Fenech, M. 2007. Buccal micronucleus cytome biomarkers may be associated with Alzheimer's disease. Mutagenesis, 22(6):371-9.

Thomas, P., Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., and Fenech M. 2009. Buccal micronucleus cytome assay. Nature Protocols, 4(6): 825-37.

Thomson, E.J., Shackleton, S., and Harrington, J.M. 1984. Chromosome aberrations and sister-chromatid exchange frequencies in pathology staff occupationally exposed to formaldehyde. Mutation Research, 141(2):89-93.

Thompson, L.H., and West, M.G. 2000. XRCC1 keeps DNA from getting stranded. Mutation Research, 459:1–18.

Thompson, L.H., and Schild, D. 2001. Homologous recombinational repair of DNA ensures mammalian chromosome stability. Mutation Research, 477(1-2):131-53.

Thompson, C.M., and Grafström, R.C. 2009. Commentary: mechanistic considerations for associations between formaldehyde exposure and nasopharyngeal carcinoma. Environmental Health, 8:53.

To-Figueras, J., Gené, M., Gómez-Catalán, J., Galán, M.C., Fuentes, M., Ramón, J.M., Rodamilans, M., Hugue,t E., and Corbella, J. 1997. Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) polymorphisms and lung cancer risk among Northwestern Mediterraneans. Carcinogenesis, 18(8): 1529-33.

Tolbert P.E., Shy C.M., Allen J.W. 1992. Micronuclei and other nuclear anomalies in buccal smears: methods development. Mutation Research, 271: 69-77.

Tollerud, .DJ., Ildstad, S.T., Brown, L.M., Clark, J.W., Blattner, W.A., Mann, D.L., Neuland, C.Y., Pankiw-Trost, L., and Hoover, R.N. 1990. T-cell subsets in healthy teenagers: transition to the adult phenotype. Clinical Immunology Immunopathology, 56(1):88-96.

Tryphonas, H., Fournier, M., Blakley, B., Smits, J., and Brousseau, P. 2005. Structural and functional complexity of the immune system and its relationship to immunotoxicology. In: Investigative Immunotoxicology, Taylor and Francis, Boca Raton, 3-10 pp.

Tuimala, J., Szekely, G., Gundy, S., Hirvonen, A., and Norppa H. 2002. Genetic polymorphisms of DNA repair and xenobiotic-metabolizing enzymes: role in mutagen sensitivity. Carcinogenesis, 23:1003–8.

Tulinska, J., Jahnova, E., Dusinska, M., Kuricova, M., Liskova, A., Ilavska, S., Horvathova, M., Wsolova, L., Kyrtopoulos, S., Collins, A., Harrington, V., and Fuortes, L. 2004. Immunomodulatory effects of mineral fibres in occupationally exposed workers. Mutation Research, 553: 111-124.

Tunca, B.T., and Egeli, U. 1996. Cytogenetic findings on shoe workers exposed long-term to benzene. Environmental Health Perspectives, 104(Suppl 6):1313-7.

Tyihák, E., Bocsi, J., Timár, F., Rácz, G., and Szende, B. 2001. Formaldehyde promotes and inhibits the proliferation of cultured tumour and endothelial cells. Cell Proliferation, 34(3):135-41.

Vahteristo, P., Yliannala, K., Tamminen, A., Eerola, H., Blomqvist, C., Nevanlinna, H. 2006. BACH1 Ser919Pro variant and breast cancer risk. BMC Cancer, 24;6:19.

Vas, G., and Vekey, K. 2004. Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis. Journal Mass Spectrometry, 39(3): 233-254.

Vasudeva, N., and Anand, C. 1996. Cytogenetic evaluation of medical students exposed to formaldehyde vapor in the gross anatomy dissection laboratory. Journal of American College Health, 44(4):177-9.

Vaughan, T.L., Strader, C., Davis, S., and Daling, J.R. 1986. Formaldehyde and cancers of the pharynx, sinus and nasal cavity: I. Occupational exposures. International journal of cancer, 38(5):677-83 [abstract].

Vaughan, T.L., Stewart, P.A., Teschke, K., Lynch, C.F., Swanson, G.M., Lyon, J.L., and Berwick, M. 2000. Occupational exposure to formaldehyde and wood dust and nasopharyngeal carcinoma. Occupational and environmental medicine, 57(6):376-84.

Veraldi, A., Costantini, A.S., Bolejack, V., Miligi, L., Vineis, P., and van Loveren, H. 2006. Immunotoxic effects of chemicals: A matrix for occupational and environmental epidemiological studies. American Journal of Industrial Medicine, 49(12):1046-55.

Vial T, Nicolas B, Descotes J. 1996. Clinical immunotoxicity of pesticides. Journal Toxicology Environmental Health, 48(3):215-29.

Viegas, S. and Prista, J. 2007. Cancro Nasofaringeo e Exposição a Formaldeído: avaliação da história profissional em 63 casos registados. Revista Saúde and Trabalho, 6:13-24.

Viegas, S., Ladeira, C., Nunes, C., Malta-Vacas, J., Gomes, M., Brito, M., Mendonca, P., and Prista, J. 2010. Genotoxic effects in occupational exposure to formaldehyde: A study in anatomy and pathology laboratories and formaldehyde-resins production. Journal of Occupational Medicine and Toxicology, 5(25):1-8.

Vineis, P., Manuguerra, M., Kavvoura, F.K., Guarrera, S., Allione, A., Rosa, F., Di Gregorio, A., Polidoro, S., Saletta, F., Ioannidis, J.P., and Matullo, G. 2009. A field synopsis on low-penetrance variants in DNA repair genes and cancer susceptibility. Journal of the National Cancer Institute, 101(1):24-36.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. 2008. Functions of natural killer cells. Nature Immunology, 9(5):503-10.

Vondracek, M., Xi, Z., Larsson, P., Baker, V., Mace, K., Pfeifer, A., Tjälve, H., Donato, M.T., Gomez-Lechon, M.J., and Grafström, R.C. 2001. Cytochrome P450 expression and related metabolism in human buccal mucosa. Carcinogenesis, 22(3):481-8.

Walrath J, Fraumeni JF, Jr. 1983. Mortality patterns among embalmers. International Journal Cancer, 31(4): 407-411[abstract].

Wang, R.S., Nakajima, T., Kawamoto, T., and Honma, T. 2002. Effects of aldehyde dehydrogenase-2 genetic polymorphisms on metabolism of structurally different aldehydes in human liver. Drug Metabolisn Disposition, 30(1):69-73.

Wang, Y., Spitz, M.R., Zhu, Y., Dong, Q., Shete, S., and Wu, X. 2003. From genotype to phenotype: correlating XRCC1 polymorphisms with mutagen sensitivity. DNA Repair, 2(8):901-8.

Wang, X., Tomso, D.J., Liu, X., and Bell, D.A. 2005. Single nucleotide polymorphism in transcriptional regulatory regions and expression of environmentally responsive genes. Toxicology Applied Pharmacology, 207(2 Suppl):84-90.

Wang, B., and Liu, D.D. 2006. Detection of formaldehyde induced developmental toxicity assessed with single cell gel electrophoresis. Fen Zi Xi Bao Sheng Wu Xue Bao 39(5):462-6 [abstract].

Wang, W., Xu, J., Xu, L., Yue, B., and Zou, F. 2007. The instability of (GpT)n and (ApC)n microsatellites induced by formaldehyde in Escherichia coli. Mutagenesis, 22(5): 353-357.

Wang, M., Cheng, G., Balbo, S., Carmella, S.G., Villalta, P.W., Hecht, S.S. 2009. Clear differences in levels of a formaldehyde-DNA adduct in leukocytes of smokers and nonsmokers. Cancer Research, 69(18):7170-4.

Wang, S.S., Bratti, M.C., Rodríguez, A.C., Herrero, R., Burk, R.D., Porras, C., González, P., Sherman, M.E., Wacholder, S., Lan, Z.E., Schiffman, M., Chanock, S.J., and Hildesheim, A. 2009. Common variants in immune and DNA repair genes and risk for human papillomavirus persistence and progression to cervical cancer. Journal Infectious Disease, 199(1):20-30.

Wang, Y., Yang, H., Li, L., Wang, H., Xia, X., and Zhang, C. 2012. Biomarkers of chromosomal damage in peripheral blood lymphocytes induced by polycyclic aromatic hydrocarbons: a meta-analysis. International archives of occupational and environmental health 85(1):13-25.

Wei, C.N., Harada, K., Ohmori, S., Wei, Q.J., Minamoto, K., and Ueda, A. 2007. Subjective symptoms of medical students exposed to formaldehyde during a gross anatomy dissection course. International journal of immunopathology and pharmacology, 20(2):23-25.

Wei, Q., Cheng, L., Amos, C.I., Wang, L.E., Guo, Z., Hong, W.K., and Spitz, and M.R. 2000a. Repair of tobacco carcinogen-induced DNA adducts and lung cancer risk: a molecular epidemiologic study. Journal of the National Cancer Institute, 92(21):1764-72.

Wei, Q., Frazier, M.L., and Levin, B. 2000b. DNA repair: a double-edged sword. Journal of the National Cancer Institute, 92(6):440-1.

West S, Hildesheim A, Dosemeci M. 1993. Non-viral risk factors for nasopharyngeal carcinoma in the Philippines: results from a case-control study. International journal of cancer, 55(5): 722-727 [abstract].

Wilson, D.M. 3rd, and Thompson, L.H. 2007. Molecular mechanisms of sister-chromatid exchange. Mutation Research, 616(1-2):11-23.

Wilson, D.M. 3rd, Kim, D., Berquist, B.R., and Sigurdson, A.J. 2011. Variation in base excision repair capacity. Mutation Research, 711(1-2):100-12.

WHO (World Health Organization). 2000. Air Quality Guidelines for Europe, 2nd edition. WHO Regional Publications, European series No. 91, Copenhagen, 273 pp.

Wu, X., Shi, H., Jiang, H., Kemp, B., Hong, W.K., Delclos, G.L., and Spitz, M.R. 1997. Associations between cytochrome P4502E1 genotype, mutagen sensitivity, cigarette smoking and susceptibility to lung cancer. Carcinogenesis, 18(5):967-73.

Wu PA, Loh CH, Hsieh LL, Liu TY, Chen CJ, Liou SH. 2004. Clastogenic effect for cigarette smoking but not areca quid chewing as measured by micronuclei in exfoliated buccal mucosal cells. Mutation Research, 562(1-2):27-38.

Yager, J.W., Cohn, K.L., Spear, R.C., Fisher, J.M., and Morse, L. 1986. Sister-chromatid exchanges in lymphocytes of anatomy students exposed to formaldehyde-embalming solution. Mutation Research, 174(2): 135-9.

Yan J, Greer JM, Hull R, O'Sullivan JD, Henderson RD, Read SJ, and McCombe PA. 2010. The effect of ageing on human lymphocyte subsets: comparison of males and females. Immunity and Ageing, 7:4.

Yang, X., Qiu, M.T., Hu, J.W., Wang, X.X., Jiang, F., Yin, R., and Xu, L. 2013. GSTT1 null genotype contributes to lung cancer risk in asian populations: a meta-analysis of 23 studies. PLoS One, 8(4):e62181.

Ye, X., Yan, W., Xie, H., Zhao, M., Ying, C. 2005. Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. Mutation Research, 588: 22-27.

Ye, Z., and Song, H. 2005. Glutathione s-transferase polymorphisms (GSTM1, GSTP1 and GSTT1) and the risk of acute leukaemia: a systematic review and meta-analysis. European Journal Cancer, 41(7):980-9.

Ying, C.J., Yan, W.S., Zhao, M.Y., Ye, X.L., Xie, H., Yin, S.Y. and Zhu, X.S. 1997. Micronuclei in nasal mucosa, oral mucosa and lymphocytes in students exposed to formaldehyde vapor in anatomy class. Biomedical and Environmental Sciences, 10: 451-455.

Ying, C.J., Ye, X.L., Xie, H., Yan, W.S., Zhao, M.Y., Xia, T., and Yin, S.Y. 1999. Lymphocyte subsets and sister-chromatid exchanges in the students exposed to formaldehyde vapor. Biomedical and Environmental Sciences, 12(2): 88-94.

Yuan L, Meng L, Ma W, Xiao Z, Zhu X, Feng JF, Yu H, Xiao R. 2011. Impact of apple and grape juice consumption on the antioxidant status in healthy subjects. International Journal Food Science Nutrition, 62(8):844-50.

Yu, H., Ma, H., Yin, M., and Wei, Q. 2012. Association between PARP-1 V762A polymorphism and cancer susceptibility: a meta-analysis. Genetic Epidemiology, 36(1):56-65.

Yu, K.D., Chen, A.X., Qiu, L.X., Fan, L., Yang, C., Shao, Z.M. 2010.XRCC2 Arg188His polymorphism is not directly associated with breast cancer risk: evidence from 37,369 subjects. Breast Cancer Research Treatment, 123(1):219-25.

Yu L.Q., Jiang S.F., Leng S.G., He F.S., and Zheng Y.X. 2005. Early genetic effects on workers occupationally exposed to formaldehyde. Chinese Journal of Preventive Medicine, 39(6): 392-5 [abstract].

Zhang, L., Steinmaus, C., Eastmond, D.A., Xin, X.K., and Smith, M.T. 2009. Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms. Mutation Research, 681(2-3):150-68.

Zhang, L., Freeman, L.E., Nakamura, J., Hecht, S.S., Vandenberg, J.J., Smith, M.T., and Sonawane, B.R. 2010b. Formaldehyde and leukemia: epidemiology, potential mechanisms, and implications for risk assessment. Environmental and Molecular Mutagenesis, 51(3):181-91.

Zhang, L., Tang, X., Rothman, N., Vermeulen, R., Ji, Z., Shen, M., Qiu, C., Guo, W., Liu, S., Reiss, B., Freeman, L.B., Ge, Y., Hubbard, A.E., Hua, M., Blair, A., Galvan, N., Ruan, X., Alter, B.P., Xin, K.X., Li, S., Moore, L.E., Kim, S., Xie, Y., Hayes, R.B., Azuma, M., Hauptmann, M., Xiong, J., Stewart, P., Li, L., Rappaport, S.M., Huang, H., Fraumeni, J.F. Jr, Smith, M.T., and Lan, Q. 2010b. Occupational exposure to formaldehyde, hematotoxicity, and leukemia-specific chromosome changes in cultured myeloid progenitor cells. Cancer Epidemiology, Biomarkers and Prevention, 19(1):80-8.

Zhong, W., and Hee, S.Q. 2004. Quantitation of normal and formaldehyde-modified deoxynucleosides by high-performance liquid chromatography/UV detection. Biomedical Chromatography, 18(7):462-9.

Zhou, D.X., Qiu,, S.D., Zhang, J., Tian, H., and Wang, H.X. 2006. The protective effect of vitamin E against oxidative damage caused by formaldehyde in the testes of adult rats. Asian Journa Andrology, 8(5):584-8.

Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D.C., Lukas, J., Bekker-Jensen, S., Bartek, J., and Shiloh, Y. 2006. Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. Nature Cell Biology, 8(8):870-6.

V. ANNEXES

ANNEX I

Ministério da Saúde
Centro de Saúde Pública Doutor Gonçalves Ferreira
Rua Alexandre Herculano, 321 - 4000-055 Porto P o r t u g a l http: www.insa.pt cgf@insa.min-saude.pt
telf. +351 223 401 100 fax. +351 223 401 109
Instituto_Nacional de Saúde Doutor Ricardo Jorge

AVALIAÇÃO DE RISCO DA EXPOSIÇÃO AO FORMALDEÍDO. ANÁLISE DO DANO GENÉTICO E SUSCEPTIBILIDADE GENÉTICA EM PROFISSIONAIS DE ANATOMIA PATOLÓGICA.

Declaração de Consentimento

Fui informado sobre os objectivos do estudo e compreendi com clareza o que me é pedido, como participante.

Fui ainda informado que:

1- Os dados que fornecer, bem como os resultados das análises que forem efectuadas sobre as amostras do meu sangue, urina e esfregaço bucal serão estritamente confidenciais. Assim que os procedimentos do estudo o permitam esses dados e resultados serão tornados anónimos, isto é, deixarão de poder ser relacionados com a minha identificação;
2- Todos os investigadores e técnicos envolvidos neste estudo e que utilizam esses dados são afectos ao Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P. (INSA, I.P.) pelo que de acordo com a política da Qualidade, Ambiente e Segurança do INSA, I.P. estão obrigados a manter a confidencialidade das informações a que tem acesso no âmbito da sua actividade.

Nestas condições, declaro que aceito participar no estudo, disponibilizando-me para:

1- Ser entrevistado e prestar informações sobre vários aspectos respeitantes a condições de saúde bem como a algumas características sócio-demográficas relevantes;
2- Permitir a colheita de uma amostra de sangue, urina e esfregaço bucal para posterior análise laboratorial.

Data:_____/_____/_____

Nome do participante:_____

Assinatura:_____

CÓDIGO DE IDENTIFICAÇÃO : _____

CÓDIGO DE IDENTIFICAÇÃO : _____

Amostra recolhida por:_____ Data:_____/_____/___ Hora:_____

A) CARACTERIZAÇÃO SÓCIO-DEMOGRÁFICA :

1. Data de Nascimento : _____/_____/_____ (Idade=_____anos)

2. Sexo: **M** □ **F** □

3. Altura: _____ Peso: _____

4. Naturalidade:_____ Localidade onde reside:_____

B) ACTIVIDADE PROFISSIONAL:

5. Actividade profissional: _____

6.1 Posto de trabalho: _____ 6.2 Há quanto tempo a exerce: _____

7. Em que consiste :_____

_____

8. Usa algum tipo de equipamento de **protecção individual**? **SIM** □ **NÃO** □
8.1 Qual?_____

9. Exerce a sua actividade profissional noutro local : **SIM** □ **NÃO** □
9.1 Quantas horas por semana ? _____

10. Profissões anteriores : _____ Período : _____

C) CARACTERIZAÇÃO DE ESTILO DE VIDA E SAÚDE:

C_1) <u>Consumo de Álcool</u>

11. Consumo .....□

Que quantidade de álcool consome em média, por dia?_____

Que tipo de bebida alcoólica bebe habitualmente? _____

12. Não consumo .....□

C_2) **Hábitos Tabágicos**

13. É **actualmente** fumador? **SIM** □ **NÃO** □ **Se é fumador:**

Com que idade começou a fumar?_____anos

Quantos cigarros fuma por dia?_____

14. Alguma vez fumou? **SIM** □ **NÃO** □ **Se é ex-fumador:**

Com que idade começou a fumar?_____anos

Com que idade deixou de fumar?_____anos

15. **Se é fumador passivo :**

Tem contacto regular durante 2 ou mais horas com fumadores?

Em casa **SIM** □ **NÃO** □

No trabalho **SIM** □ **NÃO** □

No café ou similar **SIM** □ **NÃO** □

C_3) **Historial Clínico**

16. Sofre de alergias respiratórias? **SIM** □ **NÃO** □

Se respondeu **SIM** :

16.1. Só desde que comecei a exercer a minha actual função.....□

16.2. Sempre sofri.....□

Se **sempre sofreu**, sente que houve um agravamento desde que começou a exercer a sua actual actividade profissinal ? **SIM** □ **NÃO** □

17. Costuma sentir alguma da sintomatologia seguinte?

17.1. Irritação na garganta.....□

17.2. Irritação no nariz.....□

17.3. Irritação nos olhos.....□

17.4. Aumento do lacrimejar.....□

17.5. Outros.....□ Qual/Quais ?_____

Se assinalou algum sintoma, pode especificar a altura do dia ou da semana em que sente mais?

_____

18. No último ano fez algum tipo de Exame radiológico (Raios X) ? **SIM** □ **NÃO** □
Qual?_____

19. Nos últimos 2 meses teve algum problema de saúde ? **SIM** □ **NÃO** □
Qual?_____

20. No último ano foi submetido(a) a alguma intervenção cirúrgica ? **SIM** □ **NÃO** □
Qual?_____

21. Toma algum tipo de medicação? **SIM** □ **NÃO** □
Qual/Quais ? _____

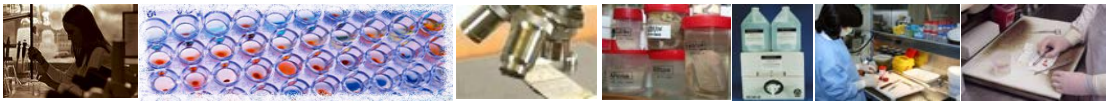
22. Alguma vez tomou ou toma pílula contraceptiva ou faz terapêutica hormonal de substituição? **SIM** □ **NÃO** □
Há quanto tempo? _____

23. Tomou alguma vacina nos últimos 12 meses? **SIM** □ **NÃO** □
Qual?_____

C_4) **Hábitos Alimentares**

24.1. Consumo de chá..... **NÃO** □ **SIM** □ Frequência _____
24.2. Consumo de café..... **NÃO** □ **SIM** □ Frequência _____
24.3. Consumo de fruta.....**NÃO** □ **SIM** □ Frequência _____
24.4. Consumo de vegetais.....**NÃO** □ **SIM** □ Frequência _____
24.5. Consumo de peixe e carne (por semana) ..... **peixe** _____ **carne** _____
24.5. Consumo de suplementos vitamínicos...**NÃO** □ **SIM** □ Frequência _____

ANNEX II



AVALIAÇÃO DO RISCO DA EXPOSIÇÃO PROFISSIONAL AO FORMALDEÍDO.

ANÁLISE DO DANO GENÉTICO E SUSCEPTIBILIDADE GENÉTICA EM PROFISSIONAIS DE ANATOMIA PATOLÓGICA

Enquadramento

O Formaldeído (FA) é um solvente orgânico largamente usado em actividades laboratoriais.

Este produto é um fixador de tecidos pouco dispendioso e bastante eficiente, sendo, por isso, o eleito em procedimentos de rotina anátomo-patológicos. É também um bom desinfectante e um meio óptimo para conservar e armazenar biópsias e peças cirúrgicas.

No Serviço de Anatomia Patológica, decorrem actividades que implicam a exposição dos trabalhadores ao FA, através da utilização e manuseamento do formol. A principal desvantagem apontada na utilização do formol é o facto de haver libertação de vapores durante o seu manuseamento, com consequente inalação por parte dos trabalhadores. Por ser um irritante primário, a sintomatologia mais comum associada à inalação de FA inclui, irritação do nariz e garganta e aumento do lacrimejar.

Recentemente, a International Agency for Research on Cancer (IARC), classificou-o agente carcinogénico humano (Grupo 1)

Objectivos

Com este trabalho pretende-se avaliar o risco da exposição ocupacional a FA em profissionais dos Serviços de Anatomia Patológica. A abordagem ao tema será múltipla, por forma a relacionar diferentes tipos de biomarcadores o que permitirá caracterizar a exposição e efeitos na saúde.

A avaliação da exposição ocupacional a FA será realizada através da medição da concentração do FA no ar (monitorização ambiental) e pela análise de diferentes indicadores biológicos (monitorização biológica). Na monitorização biológica serão estudados biomarcadores de dose interna (doseamento de FA na urina), biomarcadores de genotoxicidade (testes citogenéticos, teste do cometa) e biomarcadores de susceptibilidade genética (polimorfismos genéticos de enzimas envolvidas no metabolismo do FA e de reparação de lesões no ADN).

O conjunto de dados obtidos contribuirão para um melhor conhecimento da relação entre os níveis ambientais do FA e os consequentes efeitos biológicos adversos na saúde humana. A informação recolhida será um contributo para as entidades encarregues de definir os níveis aceitáveis para a exposição humana a FA e para os serviços que têm a seu cargo a vigilância da saúde dos trabalhadores.

ANNEX III

Table I. Genotype frequencies of studied polymorphisms coding for xenobiotic-metabolic enzymes among Caucasian and Asians.

Gene	Ethnicity	Genotypes	Frequency (%)	*References*
CYP2E1 (rs6413432)	Caucasians	T/T T/A A/A	78.9 19.3 1.8	*Teixeira et al (2004)*
	Asians	T/T T/A A/A	54.5 36.4 9.1	*SNPedia **
GSTT1 *(delection)*	Caucasians	*Present* *Null*	84.0 16.0	*Costa et al (2012)*
	Asians	*Present* *Null*	44.5 55.5	*Raimondi et al (2006)*
GSTM1 *(delection)*	Caucasians	*Present* *Null*	46.1 53.9	*Lopez-Címa et al (2012)*
	Asians	*Present* *Null*	46.2 53.8	*Cho et al (2005)*
GSTP1 *(rs1695)*	Caucasians	Ile/ Ile Ile/Val Val/Val	41.9 46.4 11.7	*Lopez-Címa et al (2012)*
	Asians	Ile/ Ile Ile/Val Val/Val	70.6 25.5 2.8	*Cho et al (2005)*

* 1000 Genomes Project, data released on 13 December 2013; Cariaso and Lennon (2012).

Table II. Frequencies of polymorphic genes related to DNA-repair pathways among racial groups.

Gene	Ethnicity	Genotypes	Frequency (%)	*References*
XRCC1 (194) (rs1799782)	Caucasians	*Arg/Arg*	89.0	*Santonocito et al (2012)*
		Arg/Trp	10.0	
		Trp/Trp	1.0	
	Asians	*Arg/Arg*	78.7-36.8	*Hung et al (2005)*
		Arg/Trp	18.7-50.0	
		Trp/Trp	2.6-13.2	
XRCC1 (399) (rs25487)	Caucasians	*Gln/Gln*	42.2	*Garcia-Léston et al (2012)*
		Gln/Arg	44.6	
		Arg/Arg	13.3	
	Asians	*Gln/Gln*	56.6	*SNPedia **
		Gln/Arg	36.7	
		Arg/Arg	6.6	
PARP1 (rs1136410)	Caucasians	*Val/Val*	77.0	*Figueiroa et al (2007)*
		Val/Ala	21.0	
		Ala/Ala	2.0	
	Asians	*Val/Val*	32.5	*Stern et al (2007)*
		Val/Ala	48.1	
		Ala/Ala	0.2	
MUTYH (rs3219489)	Caucasians	*Gln/Gln*	57.8	*SNPedia **
		Gln/His	36.1	
		His/His	6.1	
	Asians	*Gln/Gln*	33.5	*SNPedia **
		Gln/His	49.0	
		His/His	17.5	
RAD51 (rs1801321)	Caucasians	*G/G*	30.6	*Silva et al (2010)*
		G/T	50.2	
		T/T	19.2	
	Asians	*G/G*	86.4	*SNPedia **
		G/T	12.9	
		T/T	0.7	
XRCC2 (rs3218536)	Caucasians	*Arg/Arg*	81.2	*Silva et al (2010)*
		Arg/His	18.8	
		His/His	-	
	Asians	*Arg/Arg*	100	*SNPedia **
		Arg/His	-	
		His/His	-	
XRCC3 (rs861539)	Caucasians	*Thr/Thr*	37.9	*Iarmocovai et al (2006)*
		Thr/Met	51.7	
		Met/Met	10.4	
	Asians	*Thr/Thr*	82.2	*SNPedia **
		Thr/Met	17.5	
		Met/Met	0.3	
FANCA (rs7190823)	Caucasians	*Thr/Thr*	17.9	*SNPedia **
		Thr/Al	43.8	
		Ala/Ala	38.3	
	Asians	*Thr/Thr*	97.2	*SNPedia **
		Thr/Al	2.8	
		Ala/Ala	-	
BRIP1 (rs4986764)	Caucasians	*Ser/Ser*	17.0	*Frank et al (2007)*
		Ser/Pro	51.3	
		Pro/Pro	31.7	
	Asians	*Ser/Ser*	5.3	*SNPedia **
		Ser/Pro	33.9	
		Pro/Pro	60.8	

* 1000 Genomes Project, data released on 13 December 2013; Cariaso and Lennon (2012).

PUBLICAÇÕES DE SUPORTE À TESE DE DOUTORAMENTO

Toxicology 252 (2008) 40–48

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde

Solange Costa[a,b,*], Patrícia Coelho[a], Carla Costa[a], Susana Silva[a], Olga Mayan[a], Luís Silva Santos[c,d], Jorge Gaspar[c], João Paulo Teixeira[a]

[a] National Institute of Health, Environmental Health Department, Praça Coronel Pacheco 15, 4050-453 Porto, Portugal
[b] Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Rua Dr António Bernardino de Almeida, 4200-072 Porto, Portugal
[c] Faculty of Medical Sciences UNL, Department of Genetics, Rua da Junqueira 96, 1349-008 Lisboa, Portugal
[d] Centro Regional das Beiras, Universidade Católica Portuguesa, Estrada da Circunvalação, 3504-505 Viseu, Portugal

ARTICLE INFO

Article history:
Received 7 July 2008
Received in revised form 20 July 2008
Accepted 21 July 2008
Available online 31 July 2008

Keywords:
Formaldehyde
Pathology anatomy workers
Micronuclei
Sister chromatid exchanges
Comet assay

ABSTRACT

Formaldehyde (FA) is a chemical traditionally used in pathology and anatomy laboratories as a tissue preservative. Several epidemiological studies of occupational exposure to FA have indicated an increased risk of nasopharyngeal cancers in industrial workers, embalmers and pathology anatomists. There is also a clear evidence of nasal squamous cell carcinomas from inhalation studies in the rat. The postulated mode of action for nasal tumours in rats was considered biologically plausible and considered likely to be relevant to humans. Based on the available data IARC, the International Agency for Research on Cancer, has recently classified FA as a human carcinogen. Although the *in vitro* genotoxic as well as the *in vivo* carcinogenic potentials of FA are well documented in mammalian cells and in rodents, evidence for genotoxic effects and carcinogenic properties in humans is insufficient and conflicting thus remains to be more documented. To evaluate the genetic effects of long-term occupational exposure to FA a group of 30 Pathological Anatomy laboratory workers was tested for a variety of biological endpoints, cytogenetic tests (micronuclei, MN; sister chromatid exchange, SCE) and comet assay. The level of exposure to FA was evaluated near the breathing zone of workers, time weighted average of exposure was calculated for each subject. The association between the biomarkers and polymorphic genes of xenobiotic metabolising and DNA repair enzymes was also assessed. The mean level of exposure was 0.44 ± 0.08 ppm (0.04–1.58 ppm). MN frequency was significantly higher ($p = 0.003$) in the exposed subjects (5.47 ± 0.76) when compared with controls (3.27 ± 0.69). SCE mean value was significantly higher ($p < 0.05$) among the exposed group (6.13 ± 0.29) compared with control group (4.49 ± 0.16). Comet assay data showed a significant increase ($p < 0.05$) of TL in FA-exposed workers (60.00 ± 2.31) with respect to the control group (41.85 ± 1.97). A positive correlation was found between FA exposure levels and MN frequency ($r = 0.384$, $p = 0.001$) and TL ($r = 0.333$, $p = 0.005$). Regarding the genetic polymorphisms studied, no significant effect was found on the genotoxic endpoints. The results of the present biomonitoring study emphasize the need to develop safety programs.

1. Introduction

Formaldehyde (FA) is an important industrial compound with numerous applications ranging from the production of resins to medicine. At room temperature, it is a flammable and colorless gas with a strong pungent odor. It is also a naturally occurring biological compound present in all cells, tissues and body fluids. The highest level of human exposure to this aldehyde occurs in occupational settings. Because of its widespread use a relatively large number of workers are exposed to FA. Increased incidences of nasopharyngeal cancer were found in populations occupationally exposed to FA (Blair et al., 1990; Partanen, 1993; Armstrong et al., 2000; Vaughan et al., 2000; Hildesheim et al., 2001; Coggon et al., 2003; Hauptmann et al., 2004). Animal studies demonstrate that high concentrations of FA can cause irreversible damage to the nasal epithelium of rats and that in some cases rats exposed to these concentrations developed neoplasia (Merk and Speit, 1998).

* Corresponding author at: National Institute of Health, Environmental Health Department, Praça Coronel Pacheco 15, 4050-453 Porto, Portugal.
Tel.: +351 222 046 405; fax: +351 222 046 409.
E-mail addresses: solange.costa2@gmail.com, solange_costa2@yahoo.co.uk (S. Costa).

0300-483X/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2008.07.056

Based on available data the International Agency for Research on Cancer (IARC) classified FA as carcinogenic to humans (group 1) (IARC, 2006). Epidemiological studies of industrial workers, embalmers and pathology anatomists have associated FA exposure with elevated risks for cancers at various sites, including nasal cavities (Blair et al., 1990; Luce et al., 2002; Coggon et al., 2003), lung (Gardner et al., 1993; Coggon et al., 2003), brain (Hayes et al., 1990; Coggon et al., 2003), pancreas (Stone et al., 2001) and lymphohematopoietic system (Hall et al., 1991; Hauptmann et al., 2003; Pinkerton et al., 2004), however, these positive findings may have been confounded by concomitant exposures and remain controversial (Collins et al., 2001; Heck and Casanova, 2004; Marsh and Youk, 2004, 2005; Golden et al., 2006). Nevertheless, it is still unclear whether these tumours arose through a genotoxic mechanism and/or as a consequence of cytotoxicity except for carcinogenesis in nasal tissues where FA seems to proceed both by genotoxic and cytotoxic effects (Orsière et al., 2006). Several studies have shown the potential genotoxic effect of FA in proliferating cultured mammalian cell lines and in human lymphocytes. Induction of micronucleus (MN) (Suruda et al., 1993; He et al., 1998; Orsière et al., 2006), sister chromatid exchange (SCE) (He et al., 1998; Shaham et al., 1997, 2002), chromosomal aberrations (CA) (He et al., 1998), DNA-protein crosslinks (DPC) (Shaham et al., 1996, 1997, 2003) and DNA damage (comet assay) (Yu et al., 2005) were reported in industrial workers, embalmers and pathology anatomists exposed to FA.

Occupational exposure to FA occurs mainly in pathologic anatomy laboratories where it is used as a cytological fixative to preserve the integrity of cell structures for a wide variety of purposes, including cell, tissue and organelle descriptive examinations, clinical diagnoses and developmental studies. In these settings, absorption of FA occurs mainly through inhalation. Inhaled FA primarily affects the upper airways; the severity and extent of physiological response depends on its concentration in the air. Several studies have consistently shown that the levels of airborne FA in anatomy laboratories exceed recommended exposure criteria (Shaham et al., 2002; Akbar-Khanzadeh and Pulido, 2003). Such results along with the recent implications of human carcinogenicity, point out for the need of close monitoring of FA exposures. A wide range of methods is currently used for the detection of early biological effects of DNA-damaging agents in occupational settings. Assays measuring micronucleus (MN) and sister chromatid exchanges in peripheral blood lymphocytes are well-established cytogenetic techniques that have been used extensively for assessing DNA damage at the chromosomal level in human biomonitoring (Carrano and Natarajan, 1988; Fenech, 1993). Recently it has been reported that the MN test is also predictive of cancer risks in human populations (Bonassi et al., 2007). In recent years, single cell gel electrophoresis or comet assay has been proven to be a very sensitive method of human biomonitoring for the detection of levels of DNA damage, and as a useful tool for detection of genetic damage at the individual cell level (Collins, 2004). The simultaneous use of cytogenetic tests and comet assay in occupational studies allows a comparison of the presence of DNA strand breaks, due to both acute and chronic exposure, and of chromosome damage, due to clastogenic and aneugenic events. In the case of chronic exposure, comparing the levels of damage from a cytogenetic technique with the comet assay could provide information about concurrent versus past exposure (Albertini et al., 2000).

Polymorphic genes involved in the metabolism of xenobiotics may modulate the levels of biomarkers arising from environmental and/or occupational exposure to genotoxic agents (Pavanello and Clonfero, 2000). Knowledge of the real impact of genetic polymorphisms as biomarkers of susceptibility is of key significance in understanding the processes of genetic damage involved in mutagenesis and carcinogenesis (Srám and Binková, 2000) and could help to minimize risks for susceptible subjects (Laffon et al., 2006). Glutathione S-transferases (GSTs) are a superfamily of polymorphic enzymes involved in the conjugation of reactive chemical intermediates to soluble forms, and they play an important role in the detoxification of endogenous and exogenous compounds. The polymorphic genes *GSTM1* and *GSTT1* code for GSTs that are involved in the detoxification of a variety of potentially carcinogenic compounds, including polyaromatic hydrocarbon diol-epoxides, steroids and genotoxic lipoperoxidation products (Strange et al., 2001). The *GSTM1* and *GSTT1* polymorphisms are the result of the deletion of part of the gene, and this leads to the absence of activity in individuals homozygous for the deletion (null genotype) (Strange et al., 2001). The *GSTM1* and *GSTT1* null genotypes have been associated with increased risk for several cancers including, lung and colorectal cancer (Raimondi et al., 2006; Hosgood et al., 2007; Loft et al., 2007; Yeh et al., 2007). DNA repair is a key human cellular response to DNA-damaging stimuli. DNA repair mechanisms are vital responses to multiple types of DNA damage, including the ones caused by exposure to environmental and endogenous carcinogens (Kiyohara and Yoshimasu, 2007; McWilliams et al., 2008). Most of the alterations caused, if not repaired, can result in genetic instability, mutagenesis and cell death. Thus genetic variations in DNA repair genes may modulate DNA repair capacity and, therefore influence risk for cancer development (Kiyohara et al., 2007). Polymorphisms in the NER (nucleotide excision repair) pathway have been associated with increased risk for breast cancer (Milne et al., 2006; Costa, 2007), head and neck cancer (Shen et al., 2001), lung cancer (Kiyohara and Yoshimasu, 2007; Matakidou et al., 2007), basal cell skin cancer (Winsey et al., 2000) and pancreatic adenocarcinoma (McWilliams et al., 2008).

The aim of the present study was to evaluate both DNA and cytogenetic damage in peripheral lymphocytes of FA-exposed workers in pathologic anatomy laboratories. Air monitoring was performed in order to evaluate occupational exposure to FA. A multiple approach was used in order to integrate different biomarkers (effect and susceptibility). Genetic damage was studied by means of MN, SCE and comet assay (comet tail length, TL). In addition, the influence of polymorphic genes of xenobiotic metabolising enzymes (*GSTM1*, *GSTT1*) and DNA repair enzymes (*ERCC1* (rs3212986: 196bp 3′ of STP T > G, Q504K), *ERCC4* (rs1800067: Ex8 + 31G > A R415Q) and *ERCC5* (rs17655: Ex15-344G > C, D1104H; and rs2227869: Ex8-369G > C, C529S)) on the biomarkers was also analysed.

2. Methods

2.1. Subjects

The study population consisted of 30 workers exposed to FA from four hospital pathological anatomy laboratories, located in Portugal (Oporto and Aveiro districts), and 30 non-exposed control employees, matched by age, sex, lifestyle and smoking habits, working in the same area in administrative offices and without occupational exposure history to FA. The characteristics of both groups are described in Table 1. Health conditions, medical history, medication, diagnostic tests (X-rays, etc.) and lifestyle factors were assessed by means of questionnaires. Subjects of the exposed group also gave information related to working practices such as use of protective measures, years of employment, specific symptoms related to FA-exposure and chronic respiratory diseases such as asthma and others. All subjects were fully informed about the procedures and objectives of this study and each subject prior to the study signed an informed consent form. Ethical approval for this study was obtained from the institutional Ethical Board of the National Institute of Health.

2.2. Environmental monitoring

Air sampling was performed in the workers breathing zone for representative working periods, analysis of the samples allowed the calculation of the 8-h time weighted average (TWA) level of exposure to FA for each subject. Air sampling and FA analysis were performed according to the NIOSH method no. 3500.

Table 1
Characteristics of the study population

	Control group	Exposed group
Number of subjects	30	30
Gender	19 females 11 males	21 females 9 males
Age (years)[a]	37 ± 10 (25–61)	38 ± 8 (23–53)
Years of employment[a]	–	11 ± 7 (0.5–27)
Smoking status		
Non-smokers	23 (77%)	22 (73%)
Smokers	7 (23%)	8 (27%)
No cigarettes/day[a]	14 ± 8 (6–30)	13 ± 5 (5–20)
No years smoking[a]	21 ± 17 (4–51)	14 ± 8 (3–22)

[a] Mean ± S.D.

2.3. Cytogenetic assays

Heparinized venous blood samples (10 ml), were collected between 10 and 11 a.m., from each donor, and were immediately processed for the different methodologies used in this study. Workers begin their work at 9 a.m. All samples were coded and analysed under blind conditions.

2.3.1. MN test

Aliquots of 0.5 ml of heparinized whole blood were used to establish duplicate lymphocyte cultures for cytokinesis-blocked micronucleus test, as described in Teixeira et al. (2004). Cultures were incubated at 37 °C in the dark for a total of 72 h, cytochalasin B (6 μg/ml) was added at 44 h to prevent cytokinesis. Cells were collected by centrifugation and treated twice with a mixture of RPMI (pH 7.2) supplemented with 2% fetal bovine serum. The cells were centrifuged again and submitted to a mild hypotonic treatment in a mixture of RPMI (pH 7.2):deionized water (1:4, v/v), supplemented with 2% fetal bovine serum. Then, the centrifuged cells were placed on dry slides and smears were performed. After air-drying, the slides were fixed with cold methanol:acetic acid (3:1, v/v). Air-dried slides were stained with 4% Giemsa in pH 6.8 phosphate buffer. Microscopic analyses were performed on a Nikon Eclipse E400 light microscope. To determine the total number of MN in binucleated cells, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject. MN were scored blindly by the same reader and identified according to the criteria defined by Caria et al. (1995).

2.3.2. SCE test

Lymphocyte cultures for SCE were established in duplicate as described previously (Teixeira et al., 2004) in the micronucleus assay, except for the addition of 5 μl of 5-bromo-deoxyuridine (10 μg/ml) to the culture medium. Differential chromatid staining was performed with the fluorescence-plus-Giemsa procedure (Perry and Wolff, 1974). A single observer scored 50 s division metaphases for each donor (25 from each duplicate culture) on coded slides to determine the number of SCE/cell.

2.3.3. Comet assay

Lymphocytes were isolated, in duplicate, from heparinized whole blood, by centrifugation on a Ficoll density gradient. The lymphocyte layer (buffy coat) was removed and washed three times with ice-cold phosphate buffer solution (PBS) pH 7.4, at 1000 rpm (~270 × *g*) for 10 min. Cell viability, determined by trypan blue exclusion, was higher than 80% in all cases.

The alkaline version of the comet assay was performed as described by Singh et al. (1988) with minor modifications. Briefly, cells collected by centrifugation (7500 × *g* for 3 min) and suspended in 100 μl of 0.6% low-melting-point agarose (LMA) in PBS (pH 7.4) were dropped onto a frosted slide precoated with a layer of 1% normal melting point agarose. Slides were placed on ice for 4 min and allowed to solidify. Coverslips were then removed and slides were immersed in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM TrisBase, 0.25 M NaOH, pH 10) for 1 h at 4 °C, in the dark. After lysis, slides were placed on a horizontal electrophoresis tank in an ice bath. The tank was filled with freshly made alkaline electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) to cover the slides, and they were left for 20 min in the dark to allow DNA unwinding and alkali-labile site expression. Electrophoresis was carried out for 20 min at 30 V and 300 mA (1 V/cm). The slides were then washed for 10 min with 1 ml of neutralizing solution (0.4 M TrisBase, pH 7.5). After neutralization, gels were stained with 100 μl of ethidium bromide solution (20 μg/ml) and covered with coverslips for 20 min. After staining the slides were washed twice with ice-cold bidistilled water for 20 min. Two slides were prepared for each donor and a 'blind' scorer examined 50 randomly selected cells from each slide (100 cells/donor) using a magnification of 400×. Image capture and analysis were performed with Comet Assay IV software (Perceptive Instruments). Comet tail length (TL) was the DNA damage parameter evaluated. TL (μm) indicates the extent of migration of genetic material in the direction of the anode (Singh et al., 1988) and is expected to be proportional to the level of single-strand breaks and alkali-labile sites.

2.4. Genotype analysis

Genomic DNA was obtained from heparinized whole blood samples using a commercially available kit according to the manufacturer's instructions (QIAamp DNA extraction kit; Qiagen, Hilden, Germany). Each DNA sample was stored at −20 °C until analysis.

GSTM1 and *GSTT1* genotyping for gene deletions were carried out by a multiplex PCR as described by Lin et al. (1998) with minor modifications described in Gaspar et al. (2004). DNA samples were amplified with the primers (PerkinElmer Corp): 5′-GAACTCCCTGAAAAGCTAAAGC-3′ (upstream) and 5′-GTTGGGCTCAAATATACGGTGG-3′ (downstream) for *GSTM1* which produced a 219 bp product, 5′-TCACCGGATCATGGCCAGCA-3′ (upstream) and 5′-TTCCTTACTGGTCCTCACATCTC-3′ (downstream) for *GSTT1* which produced a 459-bp product. The amplification of albumin gene with the primers 5′-GCCCTCTGCTAACAAGTCCTAC-3′ (upstream) and 5′-GCCCTAAAAAGAAAATCCCCAATC-3′ (downstream) was used as an internal control and produced a 350-bp product. PCR was performed in a final volume of 50 μl, consisting of DNA (0.1 μg), dNTP (0.2 mM each) (PerkinElmer), $MgCl_2$ (2.5 mM), each primer (1.0, 0.3 and 0.2 μM for *GSTM1*, *GSTT1* and albumin, respectively), AmplitaqGold polymerase (1.25 units) (PerkinElmer), reaction buffer and 2% DMSO. Amplification was performed with an initial denaturation at 95 °C for 12 min, followed by 35 cycles of amplification performed at 94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min, using a GeneAmp 9600 thermal cycler (PerkinElmer Corp). After electrophoretic separation (30 min, 4 V/cm) the amplified products were visualized, under ultraviolet light, in ethidium bromide stained (1 μg/ml) agarose gel (1.5%).

All the genotype determinations were carried out twice in independent experiments and all the inconclusive samples were reanalysed.

ERCC1 (rs3212986), *ERCC4* (rs1800067), *ERCC5* (rs17655 and rs2227869) polymorphisms were determined using the TaqMan SNP genotyping assay (Applied Biosystems) with corresponding codes of C__2532948_10, C__3285104_10, C__1891743_10 and C__15956775_10. The 5′-nuclease allelic discrimination assay, or TaqMan assay, is a PCR-based assay for genotyping single nucleotide polymorphisms. The PCR amplification was performed in 10 μl reactions containing 10 ng of genomic DNA, 1× SNP Genotyping Assay Mix, and 1× TaqMan Universal PCR Master Mix containing optimised buffer components and Rox reference dye. The amplification conditions consisted of an initial AmpliTaq Gold® activation at 95 °C during 10 min, followed by 40 or more amplification cycles consisting of denaturation at 92 °C for 15 s and annealing/extension at 60 °C for 1 min. Amplification was performed in the 7300 Real-Time PCR System (Applied Biosystems) and sequences were detected by the SDS-*Sequence Detection Software* (version 1.3.1). All the genotype determinations were carried out twice in independent experiments and all the inconclusive samples were reanalysed.

2.5. Statistical analysis

The distribution of variables was compared with the normal distribution by means of the Kolmogorov–Smirnov goodness-of-fit test. MN was the only parameter that departed significantly from normality and therefore the non-parametric tests Mann–Whitney *U*-test and Kruskal–Wallis test were applied to data. Thus, analyses of all the other variables were performed by use of parametric tests. The effect of occupational exposure, gender, age, tobacco smoking, and *GSTM1*, *GSTT1*, *ERCC1*, *ERCC4*, and *ERCC5* genotypes on every parameter studied was evaluated by one-way analysis of variance (ANOVA) and Student's *t*-test. The associations between two variables were analysed by Pearson's (parametric) and Spearman's (non-parametric) correlation tests. The level of significance considered was 0.05. All analyses were conducted using the SPSS for Windows statistical package, version 11.0.

3. Results

To evaluate current exposure to FA in pathological anatomy laboratories air samples were collected in the workers' breathing zone. The main FA vapor emissions occurred during the macroscopic examination of FA-preserved specimens and during the disposal of specimens and waste solutions, during these tasks the mean room FA concentrations were 1.50 and 4.43 ppm, respectively. The mean level of FA exposure of the 30 individuals studied was 0.44 ppm (0.04–1.58 ppm). The American Conference of Governmental Industrial Hygienists (ACGIH) has set for FA a ceiling limit at 0.3 ppm (ACGIH, 2008). The current Portuguese occupational exposure standard is also a 0.3 ppm ceiling limit (ceiling level), meaning the maximum safe FA-airborne concentration that should never be

Table 2
Results of MN, SCE, and comet assay in the populations examined

	Mean, MN ± S.E.	Mean, SCE ± S.E.	Mean, TL (μm) ± S.E.
Controls	3.27 ± 0.69 (0–17)	4.49 ± 0.16 (3.10–3.06)	41.85 ± 1.97 (28.85–66.52)
Exposed	5.47 ± 0.76* (1–17)	6.13 ± 0.29** (3.64–8.80)	60.00 ± 2.31** (33.76–99.09)

S.E.: mean standard error.
* $p = 0.003$.
** $p < 0.05$.

exceeded during any length of time in a workers' breathing zone. Thus, the FA air concentrations obtained in this study show that workers in pathological anatomy laboratories are exposed to levels of airborne FA that exceed recommended exposure criteria.

Effect of exposure on frequencies of cytogenetic biomarkers and on DNA damage are shown in Table 2.

For all the variables studied significant increases were found in the exposed workers as compared with the controls. MN frequency was significantly higher ($p = 0.003$) in the exposed subjects (5.47 ± 0.76) when compared with controls (3.27 ± 0.69). SCE mean value was significantly higher ($p < 0.05$) among the exposed group (6.13 ± 0.29) compared with control group (4.49 ± 0.16). Comet assay data showed significantly increase ($p < 0.05$) of comet tail length (TL) in FA-exposed workers (60.00 ± 2.31) with respect to the control group (41.85 ± 1.97). Fig. 1 shows the distribution of MN and SCE frequencies and TL in the study populations. The distributions in the exposed group are displaced to higher values of damage for the three parameters studied. Moreover the exposed individuals were the only ones to have mean frequency SCE values higher than 6.4 and mean TL values equal or higher than 70.4 μm.

A positive correlation was found between FA exposure levels and MN frequency ($r = 0.384$, $p = 0.001$) and TL ($r = 0.333$, $p = 0.005$).

The effect of gender on the parameters examined is shown in Table 3. MN frequency increased in females in both groups compared with males, but the increase was not significant. No significant association was found between males and females from both groups for SCE frequency. Regarding DNA damage, comet tail length was significantly ($p = 0.014$) increased in females (63.06 ± 2.60, $n = 21$) compared with males (52.84 ± 4.00, $n = 9$) but only in the exposed group.

In order to examine the effect of age, exposed and non-exposed individuals were divided in three groups: <30 years, 30–40 years and >40 years. No significant effect of age was found in any of the variables studied (data not shown). MN frequency increased with age in both populations, but it did not reach statistical signification.

Smoking habits did not influence MN frequency and DNA damage (TL) (Table 4). However a significant increase ($p = 0.014$) was found for SCE frequency in the control group; smokers had a higher (5.24 ± 0.39, $n = 7$) frequency of SCE than non-smokers (4.27 ± 0.15, $n = 23$). In the exposed group no significant difference was observed.

In order to analyse the effect of exposure time four groups of exposed workers were established based on their years of employment. No significant association was found between the duration of exposure and the studied biomarkers (data not shown).

Distribution of the genotype frequencies of metabolic (*GSTM1*, *GSTT1*) and DNA repair (*ERCC1*, *ERCC4*, *ERCC5*) genes in the study population is presented in Table 5.

The *GSTM1* null genotype was slightly more prevalent in controls (48%) than among the exposed workers (43%), whereas the distribution of the *GSTT1* null genotype was 17% in the exposed population and 7% in the controls. Among the whole study population, the prevalence of *GSTM1* and *GSTT1* deleted genotypes was 46% and 7%, respectively, a finding consistent with previous results described by other authors for Caucasians (To-Figueras et al., 1997; Garte et al., 2001; Raimondi et al., 2006) and Portuguese populations (Martins and Alves, 1998; Gaspar et al., 2004). The distribution of *ERCC1*, *ERCC4*, *ERCC5* frequencies in the study population were similar to the ones described in other studies for Caucasian populations (NCBI-SNP Database, 2008; SNP500Cancer Database, 2008). Allele frequencies of the different *ERCC1* genotypes obtained in the study population are similar to those found for Caucasian populations NCBI-SNP Database, 2008; SNP500Cancer Database, 2008). The distribution of *ERCC4* genotypes in the whole studied population was 78%, 20% and 2% for GG, GA and AA, respectively. These results are identical to those described for Caucasian population, GG 79%, GA 19% and AA 2% (SNP500Cancer Database, 2008). The *ERCC5* (rs17655) CC genotype frequency in all studied population was 6%, which is in accordance with the 7% frequency described for the European population (NCBI-SNP Database, 2008). Concerning the frequency of the *ERCC5* (rs2227869) genotypes among the whole population, the prevalence of *ERCC5* GG genotype was 93%, a finding consistent with the frequency registered for Caucasian populations (NCBI-SNP Database, 2008; SNP500Cancer Database, 2008).

Regarding the effect of the genetic polymorphisms studied we did not find any effect on the genotoxic endpoints.

4. Discussion

Occupational exposure to FA evaluated in the four pathologic anatomy laboratories shows that workers are exposed to high levels of FA.

Several studies (Shaham et al., 1997; Dufresne et al., 2002; Akbar-Khanzadeh and Pulido, 2003) point the anatomy laboratories

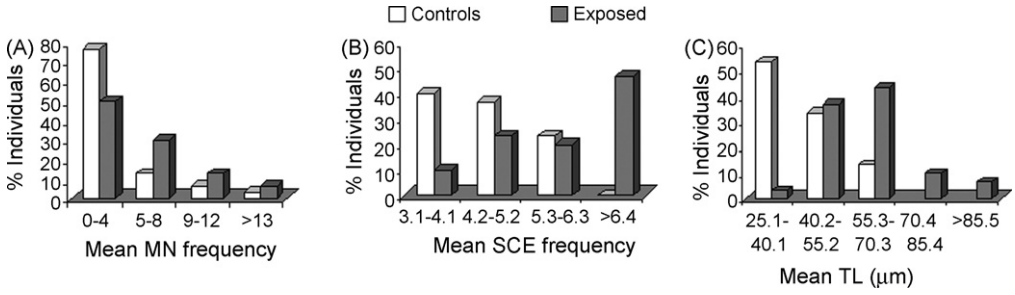


Fig. 1. Distribution of MN frequency (A), SCE frequency (B), and comet tail length (C) in control and exposed populations.

Table 3
Effect of gender on MN, SCE, and comet assay

Gender	*N*	MN ± S.E. (range)	SCE ± S.E. (range)	TL (μm) ± S.E. (range)
Controls				
Females	19	4.11 ± 1.03 (0–17)	4.33 ± 0.17 (3.27–6.16)	40.84 ± 2.75 (29.80–66.52)
Males	11	1.82 ± 0.44 (0–5)	4.78 ± 0.33 (3.10–6.04)	43.60 ± 2.58 (28.85–53.70)
Exposed				
Females	21	5.95 ± 0.80 (2–17)	6.10 ± 0.35 (3.72–8.80)	63.06 ± 2.60* (51.23–99.09)
Males	9	4.33 ± 1.72 (1–17)	6.18 ± 0.52 (3.64–8.10)	52.84 ± 4.00 (33.76–76.18)

S.E.: mean standard error.
* $p = 0.014$, significant difference with regard to males among exposed individuals.

Table 4
Effect of smoking on MN, SCE, and comet assay

Smoking habits	*N*	MN ± S.E. (range)	SCE ± S.E. (range)	TL (μm) ± S.E. (range)
Controls				
Non-smokers	23	3.09 ± 0.81 (0–17)	4.27 ± 0.15 (3.10–6.04)	40.89 ± 2.06 (29.80–62.14)
Smokers	7	3.86 ± 1.44 (0–11)	5.24 ± 0.39* (3.12–6.16)	45.03 ± 5.17 (28.85–66.52)
Exposed				
Non-smokers	22	5.35 ± 0.89 (1–17)	6.17 ± 0.32 (3.64–8.80)	59.16 ± 2.84 (33.76–99.09)
Smokers	8	5.70 ± 1.48 (2–17)	6.02 ± 0.64 (3.72–8.70)	61.66 ± 4.12 (41.01–66.10)

S.E.: mean standard error.
* $p = 0.014$, significant difference with regard to non-smokers among the controls.

as one of the occupational settings where the workers are frequently exposed to levels of FA near or superior to recommended limit values, which indicate a potential risk to workers' health. Keil et al. (2001) reported that average daily area concentrations of airborne FA in a gross anatomy laboratory ranged from 0.635 to 1.82 mg/m^3 (0.51–1.46 ppm). Shaham et al. (2002) reported for 14 pathology departments a mean low level and high level of FA exposure of 0.4 and 2.24 ppm, respectively. Ohmichi et al. (2006) evaluated personal exposure levels and indoor FA concentrations in a gross anatomy laboratory during 3 sessions of a total 20 sessions over 10 weeks. Average personal FA-exposure levels for instructors and students ranged between 0.45 and 1.08 ppm, room FA average concentrations in the three sessions were 0.45, 0.38 and 0.68 ppm.

The mean level of exposure to FA obtained in this study was 0.44 ppm (0.04–1.58 ppm). Other authors reported similar results; Akbar-Khanzadeh et al. (1994) reported for 34 workers of a gross anatomy laboratory a mean FA-level exposure of 1.24 ppm (0.07–2.94 ppm). Orsière et al. (2006) recently evaluated the occupational exposure to FA in pathology and anatomy laboratories by personal air sampling and reported a mean concentration of 0.1 ppm (0.1–0.7 ppm).

Our results show that DNA and chromosomal damage occurs in peripheral lymphocytes of pathologic anatomy laboratory workers exposed to FA. Significant increases were found in all biomarkers studied compared with control population.

SCE frequencies were higher in the FA-exposed subjects than in controls. These results agree with those presented by Shaham et al. (1997, 2002) who described a significant increase of SCE frequency in peripheral lymphocytes of 90 pathology workers from 14 hospital pathology departments. The increase of SCE on FA exposed individuals was previously described by Yager et al. (1986); SCE measured in the peripheral lymphocytes of eight non-smoking anatomy students after exposure to FA embalming solution during a 10 week anatomy class showed a significant increase ($p = 0.02$) when compared with samples obtained from the same individuals immediately before exposure began (breathing-zone samples showed a mean FA concentration of 1.2 ppm). Recently Ye et al. (2005) reported significantly increased SCE frequencies in a group of 18 workers from a FA factory. The mean exposure duration

Table 5
Frequency of metabolic and repair genotypes in the study population

Genes	DbSNP ID	Genotypes	All (%)	Controls (%)	Exposed (%)
GSTM1	Deletion	Present	32 (54)	15 (52)	17 (57)
		Null	27 (46)	14 (48)	13 (43)
GSTT1	Deletion	Present	53 (88)	28 (93)	25 (83)
		Null	7 (12)	2 (7)	5 (17)
		TT	4 (7)	2 (8)	2 (7)
ERCC1	rs3212986	TG	22 (42)	11 (46)	11 (38)
		GG	27 (51)	11 (46)	16 (55)
		GG	43 (78.2)	17 (68)	26 (87)
ERCC4	rs1800067	GA	11 (20)	8 (32)	3 (10)
		AA	1 (1.8)	0	1 (3)
		GG	32 (59)	11 (46)	21 (70)
ERCC5	rs17655	GC	19 (35)	10 (42)	9 (30)
		CC	3 (6)	3 (12)	0
ERCC5	rs2227869	GG	50 (93)	22 (92)	28 (93)
		GC	4 (7)	2 (8)	2 (7)

was 8.6 years and the level of exposure was 0.985 ± 0.286 mg/m^3 (0.82 ± 0.238 ppm). In contrast, in other studies no differences were found on SCE frequencies of subjects occupationally exposed to FA (Suruda et al., 1993, level of FA exposure was 0.33 ppm; Ying et al., 1999, FA inhalation exposure was 0.508 ± 0.299 mg/m^3).

Our results also showed a significant increase in MN frequency in workers compared with the controls. This finding is consistent with previous studies on peripheral lymphocytes and on epithelial cells (nasal and buccal) of FA exposed workers. Suruda et al. (1993) examined the effect of low level exposure to FA (0.33 ppm) on oral, nasal and lymphocyte biological markers in a group of 29 mortician students during an embalming course. Epithelial cells from the buccal mucosa showed a 12-fold increase in micronuclei frequency. Micronucleated lymphocytes frequency increased 26% and nasal epithelial micronuclei increased 22% during the study period. A dose–response relationship was observed with cumulative exposure to FA. Ying et al. (1997) evaluated micronuclei frequency on lymphocytes and on oral and nasal mucosa cells of 25 anatomy students exposed to FA over an 8-week period. A higher frequency of micronuclei was observed in nasal and oral exfoliative cells after FA exposure, but no significant increase in the frequency of lymphocyte micronuclei was found. He et al. (1998) applied cytokinesis-blocked micronucleus (CBMN) assay as a biological dosimeter to detect abnormalities in human peripheral lymphocytes of 13 students exposed to FA during a 12-week anatomy class. Breathing-zone air samples collected during dissection procedures showed a mean concentration of 2.37 ppm; MN frequencies in the exposed group were significantly increased ($p < 0.01$) compared with controls. FA-exposed group also showed a significant increase in CA ($p < 0.01$) and SCE ($p < 0.05$). A correlation between MN and CA in individuals was observed. The results indicated that FA might damage the chromosomes of human lymphocytes. Yu et al. (2005) also found significantly higher frequencies of micronuclei on peripheral lymphocytes in 151 workers occupationally exposed to FA from two plywood factories compared with control population ($p < 0.05$). Recently a study was conducted to evaluate the genotoxic effect of occupational exposure to FA on 59 pathology and anatomy laboratory workers, assessment of chromosomal damage was carried out by use of the CBMN assay in peripheral lymphocytes. The frequency of binucleated micronucleated cells was significantly higher in pathologists/anatomists than in controls ($p = 0.001$) (Orsière et al., 2006). Orsière et al. (2006) also reported a significantly higher frequency of monocentromeric micronuclei in FA-exposed pathologists/anatomists than in controls ($p < 0.001$), whereas for acentromeric micronuclei frequency no differences were obtained between the exposed and control group, suggesting an aneugenic effect of FA in peripheral lymphocytes of exposed subjects. The potential aneugenic effects of FA have also been suggested *in vitro*. Pfuhler and Wolf (2002) reported the ability of FA to disturb the kinetochore-microtubule attachment and mitotic spindle checkpoint using isolated tubulin from pig brains. On the other hand, a significantly higher fraction of acentromeric micronuclei was reported in buccal and nasal cells of mortuary science students exposed to FA before and after a 90-day embalming class (Titenko-Holland et al., 1996), suggesting a clastogenic effect of FA as the primary mechanism of micronucleus formation.

Only in the last few years the comet assay has being introduced as a useful technique in human biomonitoring studies allowing the evaluation of DNA damage at the single cell level. Therefore, few are the studies published on FA occupational exposure in which this biomarker is used. However, there are already some *in vitro* studies in cellular lines and in animal and human leukocytes culture cells in which the comet assay proved to be a sensitive biological indicator in the evaluation of the genotoxic effect of FA (Frenzilli et al., 2000; Im et al., 2006; Liu et al., 2006; Sul et al., 2007). In this study, the levels of DNA damage, measured as TL, were significantly increased in the exposed group compared with controls. This result agrees with those presented by Yu et al. (2005) who reported a significantly increase of TL and olive tail moment in peripheral blood lymphocyte of 151 workers from two plywood factories. The levels of FA-exposed workers were between 0.10 and 7.88 mg/m^3 (0.08–6.54 ppm).

In human biomonitoring studies it is important to assess the influence of major confounding factors such as gender, age and smoking habits in the endpoints studied. In this work we did not find significant differences between females and males in MN and SCE frequencies. However, females presented an increase in MN frequencies compared with males. This is in agreement with current knowledge on the effect of gender on genetic damage which determines a 1.5-fold greater MN frequency in females than in males (Fenech et al., 1999). The influence of gender in MN frequencies can be explained by supposed preferential aneugenic events involving the X-chromosome (Costa et al., 2007). Surrallés et al. (1996) reported an excessive overrepresentation of this chromosome in micronuclei of lymphocytes cultured from women. Significant increases were found for comet tail length only among exposed individuals, females had higher comet tail length than males. Although in the past the effect of gender on the level of DNA damage was regarded as a matter of controversy (Møller et al., 2000), recent studies have shown that this is not a factor that influences the damage evaluated by this indicator (Møller, 2006; Angerer et al., 2007).

Regarding the influence of age, no differences were obtained for the three biomarkers studied, although MN frequencies tended to rise with age in both groups it did not reach statistical significance. It has been postulated that the age affect in MN frequency is related to a progressive increase in spontaneous chromosome instability and the loss of efficiency in DNA repair mechanisms which may result in the accumulation of genetic lesions with increasing age (Bolognesi et al., 1999; Kirsch-Volders et al., 2006; Orsière et al., 2006).

Tobacco smoke contains a high number of mutagenic and carcinogenic substances, hence smoking is an important variable to consider in biomonitoring studies (IARC, 2002; Bonassi et al., 2003). In the present study no significant differences were obtained for MN and TL. However in both groups the smokers presented slightly higher MN frequencies and TL than non-smokers. There are some conflicting results regarding the effect of smoking on MN frequencies and comet assay parameters (Laffon et al., 2002; Bhalli et al., 2006; Ergene et al., 2007), nevertheless in two recent meta-analysis studies no association between smoking and these two genotoxic endpoints was found (Bonassi et al., 2003; Hoffmann and Speit, 2005). Significant differences were obtained in SCE frequencies among the control group. Smoking is known to increase SCE frequencies, there are numerous reports describing the effect of smoking on SCE frequency of occupationally exposed subjects (Shaham et al., 1997, 2002; Cebulska-Wasilewska et al., 1999; Rowland and Harding, 1999; Ergene et al., 2007). However it is important to note the low number of smoker subjects included in this study, which may have affected our results.

No association was found between time of exposure and the biomarkers studied. One possible explanation may be an adaptation of various systems (induction of metabolising and detoxifying enzymes, induction of DNA repair processes) over time.

In the present study, no association between *GSTM1*, *GSTT1*, *ERCC1*, *ERCC4*, and *ERCC5* polymorphisms and the three genotoxic endopoints were detected.

Genotoxicity of FA is confirmed in a variety of experimental systems ranging from bacteria to rodents. Although the positive findings from *in vivo* animal studies may provide a basis for

extrapolation to humans, the cytogenetic assays in humans have been conflicting with both positive and negative outcomes. Evidence for the genotoxic effects of FA exposure on first contact sites such as nose and respiratory tract have emerged increasingly, the biological evidence of toxicity on distant-site such as peripheral lymphocytes and bone marrow is still controversial.

Data obtained in this study indicate that genotoxic risk due to FA occupational exposure cannot be excluded. In the current FA level of exposure measured in pathologic anatomy laboratories, the DNA and chromosomal damage observed in peripheral blood lymphocytes of exposed workers might be induced by FA exposure, and be increased with the levels of exposure (positive correlation between FA exposure levels and MN frequency and TL). Although in this study the presence of centromeric signals in the MN was not assessed, the significant increase in DNA damage obtained in the comet assay (a clastogenicity assay) suggests a clastogenic mode of action of FA as the primary cause of the observed damage. Nevertheless, these results must be cautiously interpreted, owing to the relatively low number of exposed and control individuals included in this study.

The main determinants of FA exposure in the pathologic anatomy laboratories were the presence and efficiency of local exhaust systems, the efficiency of the general ventilation, the size and number of fixed anatomical parts to be handled and work methods. The highest room FA concentrations observed in this study occurred during the macroscopic examination of FA-preserved specimens and during the disposal of specimens and waste solutions. During this tasks in most of the cases the workers were not using respirators but when they used them the respirators were not the appropriate ones. The main reason presented by workers for the non-use of the respirators and goggles was the interference in the efficiency of the activities taken, namely communication difficulties, talking notes and handling material. Control aspects must take these factors into account. Implementation of security and hygiene measures in this sector as well as good practices campaigns may be crucial to decrease professional risk of FA exposed workers.

Conflict of interest

None.

Acknowledgements

Our current research is supported by Fundação Calouste Gulbenkian (grant 76436).

References

ACGIH, 2008. TLV's and BEI's—based on the Documentation of the Threshold Limit Values for Chemical Substances and Physical Agents & Biological Exposure Indices. American Conference of Governmental Industrial Hygienists, Cincinnati.

Akbar-Khanzadeh, F., Pulido, E.V., 2003. Using respirators and goggles to control exposure to air pollutants in an anatomy laboratory. Am. J. Ind. Med. 43, 326–331.

Akbar-Khanzadeh, F., Vaquerano, M.U., Akbar-Khanzadeh, M., Bisesi, M.S., 1994. Formaldehyde exposure, acute pulmonary response, and exposure control options in a gross anatomy laboratory. Am. J. Ind. Med. 26, 61–75.

Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E., Tice, R., Waters, M.D., Aitio, A., 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. Mutat. Res. 463, 111–172.

Angerer, J., Ewers, U., Wilhelm, M., 2007. Human biomonitoring: state of the art. Int. J. Hyg. Environ. Health 210, 201–228.

Armstrong, R.W., Imrey, P.B., Lye, M.S., Armstrong, M.J., Yu, M.C., Sani, S., 2000. Nasopharyngeal carcinoma in Malaysian Chinese: occupational exposures to particles, formaldehyde and heat. Int. J. Epidemiol. 29, 991–998.

Bhalli, J.A., Khan, Q.M., Nasim, A., 2006. DNA damage in Pakistani pesticide-manufacturing workers assayed using the comet assay. Environ. Mol. Mutagen. 47, 587–593.

Blair, A., Saracci, R., Stewart, P.A., Hayes, R.B., Shy, C., 1990. Epidemiologic evidence on the relationship between formaldehyde exposure and cancer. Scand. J. Work Environ. Health. 16, 381–393.

Bolognesi, C., Lando, C., Forni, A., Landini, E., Scarpato, R., Migliore, L., Bonassi, S., 1999. Chromosomal damage and ageing: effect on micronuclei frequency in peripheral blood lymphocytes. Age Ageing 28, 393–397.

Bonassi, S., et al., 2003. Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project. Mutat. Res. 543, 155–166.

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliori, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., Fenech, M., 2007. An incresead micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis 28, 625–631.

Caria, H., Chaveca, T., Laires, A., Rueff, J., 1995. Genotoxicity of quercetin in the micronucleus assay in mouse bone marrow erythrocytes, human lymphocytes, V79 cell line and identification of kinetochore-containing (CREST staining) micronuclei in human lymphocytes. Mutat. Res. 343, 85–94.

Carrano, A., Natarajan, A., 1988. Considerations for population monitoring using cytogenetic techniques. Mutat. Res.: Human Monit. (special issue) 204, 379–406.

Cebulska-Wasilewska, A., Wierzewska, A., Nizankowska, E., Graca, B., Hughes, J.A., Anderson, D., 1999. Cytogenetic damage and ras p21 oncoprotein levels from patients with chronic obstructive pulmonary disease (COPD), untreated lung cancer and healthy controls. Mutat. Res. 431, 123–131.

Coggon, D., Harris, E.C., Poole, J., Palmer, K.T., 2003. Extended follow up of a cohort of British chemical workers exposed to formaldehyde. J. Natl. Cancer Inst. 95, 1608–1615.

Collins, A.R., 2004. Comet assay for DNA damage and repair: principles, applications and limitations. Mol. Biotechnol. 26, 249–261.

Collins, J.J., Esmen, N.A., Hall, T.A., 2001. A review and meta-analysis of formaldehyde exposure and pancreatic cancer. Am. J. Ind. Med. 39, 336–345.

Costa, S., 2007. DNA repair genetic polymorphisms and breast cancer in the Portuguese population, Ph.D. Dissertation. Universidade do Minho, Braga.

Costa, C., Silva, S., Coelho, P., Roma-Torres, J., Teixeira, J.P., Mayan, O., 2007. Micronucleus analysis in a Portuguese population exposed to pesticides: preliminary survey. Int. J. Environ. Health 210, 415–418.

Dufresne, A., Infante-Rivard, C., Malo, J.L., Gautrin, D., 2002. Exposure to formaldehyde among animal health students. AIHA J (Fairfax, Va) 63, 647–650.

Ergene, S., Celik, A., Cavaş, T., Kaya, F., 2007. Genotoxic biomonitoring study of population residing in pesticide contaminated regions in Göksu Delta: micronucleus, chromosomal aberrations and sister chromatid exchanges. Environ. Int. 33, 885–887.

Fenech, M., 1993. The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. Environ. Health Perspect. 101, 101–107.

Fenech, M., Holland, N., Chang, W.P., Zeiger, E., Bonassi, S., 1999. The Human MicroNucleus Project—An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat. Res. 428, 271–283.

Frenzilli, G., Bosco, E., Barale, R., 2000. Validation of single cell gel assay in human leukocytes with 18 reference compounds. Mutat. Res. 468, 93–108.

Gardner, M.J., Pannett, B., Winter, P.D., Cruddas, A.M., 1993. A cohort study of workers exposed to formaldehyde in the British chemical industry: an update. Br. J. Ind. Med. 50, 827–834.

Garte, S., Gaspari, L., Alexandrie, A.K., Ambrosone, C., Autrup, H., Autrup, J.L., Baranova, H., Bathum, L., Benhamou, S., Boffetta, P., Bouchardy, C., Breskvar, K., Brockmoller, J., Cascorbi, I., Clapper, M.L., Coutelle, C., Daly, A., Dell'Omo, M., Dolzan, V., Dresler, C.M., Fryer, A., Haugen, A., Hein, D.W., Hildesheim, A., Hirvonen, A., Hsieh, L.L., Ingelman-Sundberg, M., Kalina, I., Kang, D., Kihara, M., Kiyohara, C., Kremers, P., Lazarus, P., Le Marchand, L., Lechner, M.C., van Lieshout, E.M., London, S., Manni, J.J., Maugard, C.M., Morita, S., Nazar-Stewart, V., Noda, K., Oda, Y., Parl, F.F., Pastorelli, R., Persson, I., Peters, W.H., Rannug, A., Rebbeck, T., Risch, A., Roelandt, L., Romkes, M., Ryberg, D., Salagovic, J., Schoket, B., Seidegard, J., Shields, P.G., Sim, E., Sinnet, D., Strange, R.C., Stücker, I., Sugimura, H., To-Figueras, J., Vineis, P., Yu, M.C., Taioli, E., 2001. Metabolic gene polymorphism frequencies in control populations. Cancer Epidemiol. Biomarkers Prev. 10, 1239–1248.

Gaspar, J., Rodrigues, S., Gil, O.M., Manita, I., Ferreira, T.C., Limbert, E., Gonçalves, L., Pina, J.E., Rueff, J., 2004. Combined effects of glutathione S-transferase polymorphisms and thyroid cancer risk. Cancer Genet. Cytogenet. 151, 60–67.

Golden, R., Pyatt, D., Shields, P.G., 2006. Formaldehyde as a potential human leukemogen: an assessment of biological plausibility. Crit. Rev. Toxicol. 36, 135–153.

Hall, A., Harrington, J.M., Aw, T.C., 1991. Mortality study of British pathologists. Am. J. Ind. Med. 20, 83–89.

Hauptmann, M., Lubin, J.H., Stewart, P.A., Hayes, R.B., Blair, A., 2003. Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries. J. Natl. Cancer Inst. 95, 1615–1623.

Hauptmann, M., Lubin, J.H., Stewart, P.A., Hayes, R.B., Blair, A., 2004. Mortality from solid cancers among workers in formaldehyde industries. Am. J. Epidemiol. 159, 1117–1130.

Hayes, R.B., Blair, A., Stewart, P.A., Herrick, R.F., Mahar, H., 1990. Mortality of U.S. embalmers and funeral directors. Am. J. Ind. Med. 18, 641–652.

He, J.L., Jin, L.F., Jin, H.Y., 1998. Detection of cytogenetic effects in peripheral lymphocytes of students exposed to formaldehyde with cytokinesis-blocked micronucleus assay. Biomed. Environ. Sci. 11, 87–92.
Heck, H., Casanova, M., 2004. The implausibility of leukemia induction by formaldehyde: a critical review of the biological evidence on distant-site toxicity. Regul. Toxicol. Pharmacol. 40, 92–106.
Hildesheim, A., Dosemeci, M., Chan, C.C., Chen, C.J., Cheng, Y.J., Hsu, M.M., Chen, I.H., Mittl, B.F., Sun, B., Levine, P.H., Chen, J.Y., Brinton, L.A., Yang, C.S., 2001. Occupational exposure to wood, formaldehyde, and solvents and risk of nasopharyngeal carcinoma. Cancer Epidemiol. Biomarkers Prev. 10, 1145–1153.
Hoffmann, H., Speit, G., 2005. Assessment of DNA damage in peripheral blood of heavy smokers with the comet assay and the micronucleus test. Mutat. Res. 581, 105–114.
Hosgood III, H.D., Berndt, S.I., Lan, Q., 2007. GST genotypes and lung cancer susceptibility in Asian populations with indoor air pollution exposures: a meta-analysis. Mutat. Res. 636, 134–143.
IARC, 2002. IARC Monographs on the Evaluation of Carcinogenic Risks to Human, Volume 83: Tobacco smoke and Involuntary Smoking, Summary of Data Reported and Evaluation. World Health Organization, Lyon.
IARC, 2006. IARC Monographs on the Evaluation of Carcinogenic Risks to Human, Volume 88: Formaldehyde, 2-Butoxyethanol and 1-*tert*-Butoxy-2-propanol. World Health Organization, Lyon.
Im, H., Oh, E., Mun, J., Khim, J.Y., Lee, E., Kang, H.S., Kim, E., Kim, H., Won, N.H., Kim, Y.H., Jung, W.W., Sul, D., 2006. Evaluation of toxicological monitoring markers using proteomic analysis in rats exposed to formaldehyde. J. Proteome Res. 5, 1354–1366.
Keil, C.B., Akbar-Khanzadeh, F., Konecny, K.A., 2001. Characterizing formaldehyde emission rates in a gross anatomy laboratory. Appl. Occup. Environ. Hyg. 16, 967–972.
Kirsch-Volders, M., Mateuca, R.A., Roelants, M., Tremp, A., Zeiger, E., Bonassi, S., Holland, N., Chang, W.P., Aka, P.V., Deboeck, M., Godderis, L., Haufroid, V., Ishikawa, H., Laffon, B., Marcos, R., Migliore, L., Norppa, H., Teixeira, J.P., Zijno, A., Fenech, M., 2006. The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo. Cancer Epidemiol. Biomarkers Prev. 15, 1038–1042.
Kiyohara, C., Yoshimasu, K., 2007. Genetic polymorphisms in the nucleotide excision repair pathway and lung cancer risk: a meta-analysis. Int. J. Med. Sci. 4, 59–71.
Laffon, B., Pásaro, E., Méndez, J., 2002. Evaluation of genotoxic effects in a group of workers exposed to low levels of styrene. Toxicology 171, 175–186.
Laffon, B., Teixeira, J.P., Silva, S., Roma-Torres, J., Pérez-Cadahía, B., Méndez, J., Pásaro, E., Mayan, O., 2006. Assessment of occupational genotoxic risk in the production of rubber tyres. Ann. Occup. Hyg. 50, 583–592.
Lin, D., Tang, Y., Peng, Q., Lu, S., Ambrosone, C., Kadublar, F., 1998. Susceptibility to esophageal cancer and genetic polymorphisms in glutathione-S-transferase T1, P1 and M1 and cytochrome P450 2E1. Cancer Epidemiol. Biomarkers Prev. 7, 1013–1018.
Liu, Y., Li, C.M., Lu, Z., Ding, S., Yang, X., Mo, J., 2006. Studies on formation and repair of formaldehyde-damaged DNA by detection of DNA-protein crosslinks and DNA breaks. Front. Biosci. 11, 991–997.
Loft, S., Svoboda, P., Kasai, H., Tjønneland, A., Møller, P., Sørensen, M., Overvad, K., Autrup, H., Raaschou-Nielsen, O., 2007. Prospective study of urinary excretion of 7-methylguanine and the risk of lung cancer: effect modification by mu class glutathione-S-transferases. Int. J. Cancer. 121, 1579–1584.
Luce, D., Leclerc, A., B'egin, D., Demers, P.A., G'erin, M., Orlowski, E., Kogevinas, M., Belli, S., Bugel, I., Bolm-Audorff, U., Brinton, L.A., Comba, P., Hardell, L., Hayes, R.B., Magnani, C., Merler, E., Preston-Martin, S., Vaughan, T.L., Zheng, W., Boffetta, P., 2002. Sinonasal cancer and occupational exposures: a pooled analysis of 12 case-control studies. Cancer Causes Control 13, 147–157.
Marsh, G.M., Youk, A.O., 2004. Reevaluation of mortality risks from leukemia in the formaldehyde cohort study of the National Cancer Institute. Regul. Toxicol. Pharmacol. 40, 113–124.
Marsh, G.M., Youk, A.O., 2005. Reevaluation of mortality risks from nasopharyngeal cancer in the formaldehyde cohort study of the National Cancer Institute. Regul. Toxicol. Pharmacol. 42, 275–283.
Martins, G., Alves, M., 1998. Glutathione S transferase mu polymorphism and gastric cancer in the Portuguese population. Biomarkers 3, 441–447.
Matakidou, A., el Galta, R., Webb, E.L., Rudd, M.F., Bridle, H., GELCAPS Consortium, Eisen, T., Houlston, R.S., 2007. Genetic variation in the DNA repair genes is predictive of outcome in lung cancer. Hum. Mol. Genet. 16, 2333–2340.
McWilliams, R.R., Bamlet, W.R., Cunningham, J.M., Goode, E.L., Andrade, M., Boardman, L.A., Petersen, G.M., 2008. Polymorphisms in DNA repair genes, smoking, and pancreatic adenocarcinoma risk. Cancer Res. 68, 4928–4935.
Merk, O., Speit, G., 1998. Significance of formaldehyde-induced DNA-protein crosslinks for mutagenesis. Environ. Mol. Mutagen. 32, 260–268.
Milne, R.L., Ribas, G., González-Neira, A., Fagerholm, R., Salas, A., González, E., Dopazo, J., Nevanlinna, H., Robledo, M., Benítez, J., 2006. ERCC4 associated with breast cancer risk: a two-stage case–control study using high-throughput genotyping. Cancer Res. 66, 9420–9427.
Møller, P., 2006. Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA. Mutat. Res. 612, 84–104.
Møller, P., Knudsen, L.E., Loft, S., Wallin, H., 2000. The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. Cancer Epidemiol. Biomarkers Prev. 9, 1005–1015.
NCBI-SNP Database. Available: http://www.ncbi.nlm.nih.gov/ (accessed on June 19, 2008).
NIOSH (National Institute for Occupational Safety and Health), 1994. Formaldehyde: Method 3500 (Issue 2). In: NIOSH (Ed.), NIOSH Manual of Analytical Method. U.S. Department of Health and Human Services, Cincinnati, Ohio, pp. 2–5.
Ohmichi, K., Komiyama, M., Matsuno, Y., Takanashi, Y., Miyamoto, H., Kadota, T., Maekawa, M., Toyama, Y., Tatsugi, Y., Kohno, T., Ohmichi, M., Mori, C., 2006. Formaldehyde exposure in a gross anatomy laboratory—personal exposure level is higher than indoor concentration. Environ. Sci. Pollut. Res. Int. 13, 120–124.
Orsière, T., Sari-Minodier, I., Iarmarcovai, G., Botta, A., 2006. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. Mutat. Res. 605, 30–41.
Partanen, T., 1993. Formaldehyde exposure and respiratory cancer—a meta-analysis of the epidemiologic evidence. Scand. J. Work Environ. Health 19, 8–15.
Pavanello, S., Clonfero, E., 2000. Biological indicators of genotoxic risk and metabolic polymorphisms. Mutat. Res. 463, 285–308.
Perry, P., Wolff, S., 1974. New Giemsa method for the differential staining of sister chromatids. Nature 251, 156–158.
Pfuhler, S., Wolf, H.U., 2002. Effects of the formaldehyde releasing preservatives dimethylol urea and diazolidinyl urea in several short-term genotoxicity tests. Mutat. Res. 514, 133–146.
Pinkerton, L.E., Hein, M.J., Stayner, L.T., 2004. Mortality among a cohort of garment workers exposed to formaldehyde: an update. Occup. Environ. Med. 61, 193–200.
Raimondi, S., Paracchini, V., Autrup, H., Barros-Dios, J.M., Benhamou, S., Boffetta, P., Cote, M.L., Dialyna, I.A., Dolzan, V., Filiberti, R., Garte, S., Hirvonen, A., Husgafvel-Pursiainen, K., Imyanitov, E.N., Kalina, I., Kang, D., Kiyohara, C., Kohno, T., Kremers, P., Lan, Q., London, S., Povey, A.C., Rannug, A., Reszka, E., Risch, A., Romkes, M., Schneider, J., Seow, A., Shields, P.G., Sobti, R.C., Sørensen, M., Spinola, M., Spitz, M.R., Strange, R.C., Stücker, I., Sugimura, H., To-Figueras, J., Tokudome, S., Yang, P., Yuan, J.M., Warholm, M., Taioli, E., 2006. Meta- and pooled analysis of GSTT1 and lung cancer: a HuGE-GSEC review. Am. J. Epidemiol. 164, 1027–1042.
Rowland, R.E., Harding, K.M., 1999. Increased sister chromatid exchange in the peripheral blood lymphocytes of young women who smoke cigarettes. Hereditas 131, 143–146.
Shaham, J., Bomstein, Y., Meltzer, A., Kaufman, Z., Palma, E., Ribak, J., 1996. DNA–protein crosslinks, a biomarker of exposure to formaldehyde—in vitro and in vivo studies. Carcinogenesis 17, 121–125.
Shaham, J., Bomstein, Y., Meltzer, A., Ribak, J., 1997. DNA-protein crosslinks and sister chromatid exchanges as biomarkers of exposure to formaldehyde. Int. J. Occup. Environ. Health. 3, 95–104.
Shaham, J., Gurvich, R., Kaufman, Z., 2002. Sister chromatid exchange in pathology staff occupationally exposed to formaldehyde. Mutat. Res. 15, 115–123.
Shaham, J., Bomstein, Y., Gurvich, R., Rashkovsky, M., Kaufman, Z., 2003. DNA–protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde. Occup. Environ. Med. 60, 403–409.
Shen, H., Sturgis, E.M., Khan, S.G., Qiao, Y., Shahlavi, T., Eicher, S.A., Xu, Y., Wang, X., Strom, S.S., Spitz, M.R., Kraemer, K.H., Wei, Q., 2001. An intronic poly (AT) polymorphism of the DNA repair gene XPC and risk of squamous cell carcinoma of the head and neck: a case–control study. Cancer Res. 61, 3321–3325.
Singh, N., McCoy, M., Tice, R., Schneider, E., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
SNP500Cancer Database. http://snp500cancer.nci.nih.gov/ (accessed on June 19, 2008).
Srám, R.J., Binková, B., 2000. Molecular epidemiology studies on occupational and environmental exposure to mutagens and carcinogens, 1997–1999. Environ. Health Perspect. 108, 57–70.
Stone, R.A., Youk, A.O., Marsh, G.M., Buchanich, J.M., McHenry, M.B., Smith, T.J., 2001. Historical cohort study of US man-made vitreous fiber production workers: IV. Quantitative exposure-response analysis of the nested case-control study of respiratory system cancer. J. Occup. Environ. Med. 43, 779–792.
Strange, R.C., Spiteri, M.A., Ramachandran, S., Fryer, A.A., 2001. Glutathione-S-transferase family of enzymes. Mutat. Res. 482, 21–26.
Sul, D., Kim, H., Oh, E., Phark, S., Cho, E., Choi, S., Kang, H.S., Kim, E.M., Hwang, K.W., Jung, W.W., 2007. Gene expression profiling in lung tissues from rats exposed to formaldehyde. Arch. Toxicol. 81, 589–597.
Surrallés, J., Falck, G., Norppa, H., 1996. In vivo cytogenetic damage revealed by FISH analysis of micronuclei in uncultured human T lymphocytes. Cytogenet. Cell Genet. 75, 151–154.
Suruda, A., Schulte, P., Boeniger, M., Hayes, R.B., Livingston, G.K., Steenland, K., 1993. Cytogenetic effects of formaldehyde exposure in students of mortuary science. Cancer Epidemiol. Biomarkers Prev. 2, 453–460.
Teixeira, J., Gaspar, J., Silva, S., Torres, J., Silva, S., Azevedo, M., Neves, P., Laffon, B., Mendez, J., Goncalves, C., Mayan, O., Farmer, P., Rueff, J., 2004. Occupational exposure to styrene: modulation of cytogenetic damage and levels of urinary metabolites of styrene by polymorphisms in genes CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1. Toxicology 195, 231–242.
Titenko-Holland, N., Levine, A.J., Smith, M.T., Quintana, P.J., Boeniger, M., Hayes, R., Suruda, A., Schulte, P., 1996. Quantification of epithelial cell micronuclei by fluorescence in situ hybridization (FISH) in mortuary science students exposed to formaldehyde. Mutat. Res. 371, 237–248.
To-Figueras, J., Gené, M., Gómez-Catalán, J., Galán, M.C., Fuentes, M., Ramón, J.M., Rodamilans, M., Huguet, E., Corbella, J., 1997. Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) polymorphisms and lung cancer risk among Northwestern Mediterraneans. Carcinogenesis 18, 1529–1533.

Vaughan, T.L., Stewart, P.A., Teschke, K., Lynch, C.F., Swanson, G.M., Lyon, J.L., 2000. Occupational exposure to formaldehyde and wood dust and nasopharyngeal carcinoma. Occup. Environ. Med. 57, 376–384.

Winsey, S.L., Haldar, N.A., Marsh, H.P., Bunce, M., Marshall, S.E., Harris, A.L., Wojnarowska, F., Welsh, K.I., 2000. A variant within the DNA repair gene XRCC3 is associated with the development of melanoma skin cancer. Cancer Res. 60, 5612–5616.

Yager, J.W., Cohn, K.L., Spear, R.C., Fisher, J.M., Morse, L., 1986. Sister-chromatid exchanges in lymphocytes of anatomy students exposed to formaldehyde-embalming solution. Mutat. Res. 174, 135–139.

Ye, X., Yan, W., Xie, H., Zhao, M., Ying, C., 2005. Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. Mutat. Res. 588, 22–27.

Yeh, C.C., Sung, F.C., Tang, R., Chang-Chieh, C.R., Hsieh, L.L., 2007. Association between polymorphisms of biotransformation and DNA-repair genes and risk of colorectal cancer in Taiwan. J. Biomed. Sci. 14, 183–193.

Ying, C.J., Yan, W.S., Zhao, M.Y., Ye, X.L., Xie, H., Yin, S.Y., Zhu, X.S., 1997. Micronuclei in nasal mucosa, oral mucosa and lymphocytes in students exposed to formaldehyde vapor in anatomy class. Biomed. Environ. Sci. 10, 451–455.

Ying, C.J., Ye, X.L., Xie, H., Yan, W.S., Zhao, M.Y., Xia, T., Yin, S.Y., 1999. Lymphocyte subsets and sister-chromatid exchanges in the students exposed to formaldehyde vapor. Biomed. Environ. Sci. 12, 88–94.

Yu, L.Q., Jiang, S.F., Leng, S.G., He, F.S., Zheng, Y.X., 2005. Early genetic effects on workers occupationally exposed to formaldehyde. Chin. J. Prev. Med. 39, 392–395.

Journal of Toxicology and Environmental Health, Part A: Current Issues
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uteh20

Cytogenetic and Immunological Effects Associated with Occupational Formaldehyde Exposure

Solange Costa [a], Julia García-Lestón [b], Marta Coelho [a], Patrícia Coelho [a c], Carla Costa [a d], Susana Silva [a], Beatriz Porto [e], Blanca Laffon [b] & João Paulo Teixeira [a c]

[a] National Institute of Health, Environmental Health Department, Porto, Portugal

[b] Toxicology Unit, Department of Psychobiology, University of A Coruña, Edificio de Servicios Centrales de Investigación, A Coruña, Spain

[c] ICETA, Institute of Agricultural and Agri-foodstuffs Sciences and Technologies, University of Porto, Porto, Portugal

[d] ISPUP, Institute of Public Health, University of Porto, Porto, Portugal

[e] ICBAS, Biomedical Sciences Institute of Abel Salazar, University of Porto, Porto, Portugal

Published online: 20 Mar 2013.

To cite this article: Solange Costa, Julia García-Lestón, Marta Coelho, Patrícia Coelho, Carla Costa, Susana Silva, Beatriz Porto, Blanca Laffon & João Paulo Teixeira (2013) Cytogenetic and Immunological Effects Associated with Occupational Formaldehyde Exposure, Journal of Toxicology and Environmental Health, Part A: Current Issues, 76:4-5, 217-229, DOI: 10.1080/15287394.2013.757212

To link to this article: http://dx.doi.org/10.1080/15287394.2013.757212

Journal of Toxicology and Environmental Health, Part A, 76:217–229, 2013

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287394.2013.757212

Taylor & Francis
Taylor & Francis Group

CYTOGENETIC AND IMMUNOLOGICAL EFFECTS ASSOCIATED WITH OCCUPATIONAL FORMALDEHYDE EXPOSURE

Solange Costa[1], Julia García-Lestón[2], Marta Coelho[1], Patrícia Coelho[1,3], Carla Costa[1,4], Susana Silva[1], Beatriz Porto[5], Blanca Laffon[2], João Paulo Teixeira[1,3]

[1]National Institute of Health, Environmental Health Department, Porto, Portugal
[2]Toxicology Unit, Department of Psychobiology, University of A Coruña, Edificio de Servicios Centrales de Investigación, A Coruña, Spain
[3]ICETA, Institute of Agricultural and Agri-foodstuffs Sciences and Technologies, University of Porto, Porto, Portugal
[4]ISPUP, Institute of Public Health, University of Porto, Porto, Portugal
[5]ICBAS, Biomedical Sciences Institute of Abel Salazar, University of Porto, Porto, Portugal

Formaldehyde (FA) is a widely used industrial chemical for which exposure is associated with nasopharyngeal and sinonasal cancer. Based on sufficient evidence of carcinogenicity from human investigations, supporting studies on mechanisms underlying carcinogenesis, and experimental evidence in animals, FA status was recently revised and reclassified as a human carcinogen. The highest level of exposure to FA occurs in occupational settings. Although several studies reported FA ability to induce genotoxic responses in exposed workers, not all findings were conclusive. In addition, published studies on the immunological effects of FA indicate that this compound may be able to modulate immune responses, although data in exposed subjects are still preliminary. In this study a group of pathology anatomy workers exposed to FA was evaluated for cytogenetic and immunological parameters. A control group with similar sociodemographic characteristics and without known occupational exposure to FA was also included. Genotoxicity was evaluated by means of micronucleus (MN) test, sister chromatid exchanges (SCE), and T-cell receptor (TCR) mutation assay. Percentages of different lymphocyte subpopulations were selected as immunotoxic biomarkers. The mean level of FA environmental exposure was 0.36 ± 0.03 ppm. MN and SCE frequencies were significantly increased in the exposed group. A significant decrease of the percentage of B cells in the exposed group was also found. Data obtained in this study indicate that genotoxic and immunotoxic increased risk due to FA occupational exposure cannot be excluded. Implementation of effective control measures along with hazard prevention campaigns may be crucial to decrease the risk.

Formaldehyde (FA) is a high-volume production chemical produced globally with a large range of industrial and medical purposes. Listed, since 2004, by IARC as a human carcinogen (group 1), FA status was recently revised by the U.S. government, which reclassified this compound as known to be a human carcinogen (group A). Both reclassifications are based on sufficient evidence of carcinogenicity from human investigations, supporting studies on mechanisms underlying carcinogenesis, and experimental evidence in animals. Numerous epidemiological studies of occupational exposed populations demonstrated a causal relationship between exposure to FA and cancer (IARC, 2006; NTP, 2010).

This work is supported by Fundação para a Ciência e a Tecnologia (FCT) under the grants SFRH/BD/46929/2008 and PTDC/SAU-ESA/102367/2008.

Address correspondence to Solange Costa, National Institute of Health, Environmental Health Department; Rua Alexandre Herculano, 321, 4000-055 Porto, Portugal. E-mail: solange.costa2@gmail.com

Consistent findings of increased risks of certain types of rare cancers, namely, nasopharyngeal and sinonasal cancer, were found among workers with higher measures of exposure to FA (exposure level or duration). The mechanisms by which FA induced cancer are not completely understood but most likely involve multiple modes of action, such as DNA reactivity, gene mutation, epigenetic effects, chromosomal breakage, aneuploidy, and oxidative stress (Lu et al., 2008; NTP, 2010). Zhang et al. (2010) suggested a potential causal association between occupational exposure to FA and excess mortality from leukemia, especially myeloid leukemia. However, due to mix results, evidence for FA leukemogenicity remains controversial (Checkoway et al., 2012). Given its economic importance and widespread use, many individuals are environmentally and/or occupationally exposed to FA. Nonoccupational exposure includes vehicles emissions, tobacco smoke, and household products. The highest level of human exposure to this aldehyde occurs in occupational settings. Occupational exposure involves not only individuals employed in the direct manufacture of FA and products containing it (Paustenbach et al., 1997), but also those using these products, such as those working in pathology anatomy labs where it is commonly used as a fixative and tissue preservative.

In the last decade a large number of toxicological studies were published regarding FA. FA-induced genotoxicity was confirmed in a variety of experimental systems ranging from bacteria to rodents. Although these positive findings may provide a basis for extrapolation to humans, the cytogenetic assays in humans have been conflicting with both positive and negative outcomes. Genotoxic endpoint analyses are of great interest in risk assessment of occupational carcinogens because they precede adverse health effects, thus offering a greater potential for preventive intervention (Mayeux, 2004). Genotoxicity evaluation constitutes a valuable tool for studying the most important occupational and environmental hazards to public health occurring in the past few decades and allows a reasonable epidemiological evaluation of cancer prediction (Bonassi et al., 2005; Laffon et al., 2006). Cytogenetic markers such as micronuclei (MN) and sister chromatid exchange (SCE) are well-established endpoints that were extensively used for assessing DNA damage at the chromosomal level in human biomonitoring studies (Carrano and Natarajan, 1988; Fenech, 1993).

Evaluation of potential adverse effects on the immune system is also an important component of the overall evaluation of a compound toxicity (Luebke et al., 2006). The immune response is a complex process involving the interaction of various components from anatomical barriers to specialized cells. This interaction among the various components of the immune system is extremely advantageous for the organism as the continuing dialogue between innate and acquired immune response, and efficiency is ensured. A number of biological or chemical agents have the ability to alter the functionality of the immune system, potentially compromising the organism's ability to recognize, control, or eliminate infectious agents or neoplastic cells (Veraldi et al., 2006). Studies on immunological effects induced by FA predominantly focused on the allergic reactions such as contact dermatitis and occupational asthma. In fact, a few investigations on immunological parameters (Tang et al., 2009, Hosgood et al. 2012) suggested that FA may alter these endpoints in exposed individuals.

The aim of the present study was to evaluate both genotoxic and immunotoxic parameters using peripheral lymphocytes of FA-exposed workers employed in pathology anatomy labs. Air sampling was performed in order to determine FA levels of exposure in each worker. Genotoxic damage was studied by means of MN test, „ and T-cell receptor (TCR) mutation assay. Percentages of major lymphocyte subsets, namely, T lymphocytes (%$CD3^+$), T-helper lymphocytes (%$CD4^+$), T-cytotoxic lymphocytes (%$CD8^+$), B lymphocytes (%$CD19^+$), and natural killer (NK) cells (%$CD16\text{-}56^+$), were selected as immunotoxicity markers.

METHODS

Subject Selection and Blood Sample Collection

The study population consisted of 35 subjects working for at least 1 yr in 4 hospital pathology anatomy labs, located in Portugal, and 35 nonexposed control employees, working in the same area in administrative offices but without occupational exposure history to formaldehyde (FA). The characteristics of both groups are described in Table 1. Relevant individual information on age, smoking habits, health conditions, medical history, medication, and diagnostic tests (x-rays etc.) was assessed by means of questionnaires. Subjects that stopped smoking for more than 2 yr were considered nonsmokers. Workers also provided information related to working practices such as use of protective measures, years of employment, specific symptoms related to FA exposure and chronic respiratory diseases and other disorders. Ethical approval for this study was obtained from the Ethical Board of the National Institute of Health. All subjects were fully informed about the procedures and aims of this study and each subject prior to the study signed an informed consent form. Peripheral blood samples were collected by venipuncture from each donor between 10 and 11 a.m. All samples were coded and analyzed under blind conditions.

Environmental Monitoring

Air sampling was performed in the workers breathing zone for representative working periods. Analysis of the samples allowed the calculation of the 8-h time-weighted average (TWA) level of exposure to FA for each subject. Air sampling and FA analysis were performed according to the NIOSH method number 3500 (NIOSH, 1994).

Micronucleus (MN) Test

Aliquots of 0.5 ml of heparinized whole blood were used to establish duplicate lymphocyte cultures for cytokinesis-blocked MN test as described by Teixeira et al. (2004). Microscopic analyses were performed using a Nikon Eclipse E400 light microscope. To determine the total number of MN in binucleated cells, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) was scored for each subject. MN were scored blindly by the same reader and identified according to the criteria defined by Fenech (2007).

Sister Chromatid Exchanges (SCE)

Lymphocyte cultures for SCE were established in duplicate as described by Teixeira et al. (2004). Differential chromatid staining

TABLE 1. Characteristics of the Study Population

	Control ($n = 35$)	Exposed ($n = 35$)	p Value
Gender			.513[b]
Females	31	28	
Males	4	7	
Age (yr)[a]	39.8 ± 10.0 (24–61)	41.2 ± 8.7 (26–56)	.527[c]
Years of employment[a]	—	12.5 ± 8.1 (1–30)	
BMI (kg/m^2)[a]	24.1 ± 4.8 (19–38)	23.4 ± 3.3 (17–32)	.464[c]
Smoking status			
Nonsmokers	28 (80%)	28 (80%)	
Smokers	7 (20%)	7 (20%)	
Packs per year[a]	13.9 ± 10.3 (2.8–32.3)	12.9 ± 11.2 (0.6–35.0)	.863[c]

[a]Mean ± SD (range).
[b]Fisher's exact test.
[c]Student's t-test.

was performed with the fluorescence-plus-Giemsa procedure (Perry and Wolff, 1974). Microscopic analyses were performed using a Nikon Eclipse E400 light microscope. A single observer scored 50 second-division metaphases for each donor (25 from each duplicate culture) on coded slides to determine the number of SCE per cell.

TCR Mutation Assay

Peripheral blood mononuclear leukocytes were isolated in BD Vacutainer Cell Preparation Tubes (CPT) with sodium heparin according to the manufacturer's instructions. After centrifugation, cells were washed thrice with ice-cold pH 7.4 phosphate buffer solution. TCR mutation assay was performed by a flow cytometric methodology according to Akiyama et al. (1995) with minor modifications (García-Lestón et al., 2011). Cell suspensions were analyzed by a FACScalibur flow cytometer with Cell Quest Pro software (Becton Dickinson). A minimum of 2.5×10^5 lymphocyte-gated events was acquired, and mutation frequencies of TCR (TCR-Mf) were calculated as the number of events in the mutant cell window ($CD3^-CD4^+$ cells) divided by the total number of events corresponding to $CD4^+$ cells.

Lymphocyte Subpopulations

Cell percentages of total T lymphocytes (%$CD3^+$), T-helper (Th) lymphocytes (%$CD4^+$), T-cytotoxic (Tc) lymphocytes (%$CD8^+$), B lymphocytes (%$CD19^+$), and natural killer (NK) cells (%CD16-56^+) were determined by flow cytometric measurements using a three-color direct immunofluorescence surface marker methodology described by García-Lestón et al. (2011). Analyses were carried out in a FACScalibur flow cytometer using Cell Quest Pro software (Becton Dickinson). After gating the lymphocytes based on forward/side scatter plots, fluorescence data from FL1 (FITC), FL2 (PE), and FL3 (PECy5) were obtained. At least 104 events in the lymphocytes window were acquired.

Statistical Analysis

All analyses were conducted using the SPSS for Windows statistical package 16.0. The statistical differences between means and the relationship between categorical variables in the characteristics of the study population were assessed by means of Student's *t*-test and Fisher's exact test, respectively. All results obtained in the study were assessed for normal distribution using the Kolmogorov–Smirnov test and graphic evaluation (histograms, Q–Q plots, P–P plots). When the assumption of normality was not fitted, data were transformed to normalize the distribution. TCR-Mf, B lymphocytes, NK cells, and Th/Tc ratio were the only parameters that departed significantly from normality and therefore these data were transformed on the natural logarithm scale (ln). The effect of exposure on the level of genotoxicity and immunological biomarkers was preliminarily tested using Student's *t*-test. Multivariant analysis was carried out to evaluate the contribution of exposure and potential confounding factors to the response variables considered. Correlation between variables was analyzed by Pearson's correlation test. The level of significance considered was .05.

RESULTS

The general characteristics of the studied population are summarized in Table 1. In total, 70 subjects (35 exposed and 35 controls) were involved in the study. Both groups were similar in gender distribution, age, body mass index (BMI), and smoking habits. The mean level of worker's exposure to formaldehyde (FA) was 0.36 ± 0.03 ppm (range 0.23–0.69 ppm). The peak emission of FA occurred during two routine tasks: macroscopic examination of FA-preserved specimens, and disposal of specimens and waste solutions. The current Portuguese occupational exposure limit is 0.3 ppm (ceiling level), indicating this is the maximum safe FA concentration that should never be exceeded during any length of time in a worker's breathing zone. The American Conference of Governmental

Industrial Hygienists (ACGIH) also set a ceiling exposure limit of 0.3 ppm (ACGIH, 2008). Our results show that workers in the pathology anatomy labs analyzed were exposed to airborne concentrations of FA that exceeded the national guideline limit value and ACGIH-recommended exposure criteria.

The genotoxicity biomarkers results are shown in Figure 1 as a univariant analysis. MN frequency was significantly 2.5-fold higher in FA-exposed workers than in unexposed individuals. In addition, SCE mean value was significantly increased in exposed group by 1.3-fold. A positive significant correlation was found between these two cytogenetic biomarkers. TCR-Mf did not differ markedly between exposed and control subjects.

Data obtained from univariant analysis of lymphocyte subpopulations in FA-exposed subjects and controls are illustrated in Figure 2. No significant differences were found for T cells—total %CD3$^+$ T lymphocytes, %CD4$^+$ T helper cells (Th), %CD8$^+$ T cytotoxic cells (Tc), Th/Tc ratio—and for %CD16-56$^+$ NK cells. However, a significant decrease of 0.7-fold in %CD19$^+$ B lymphocytes was noted in exposed individuals.

Table 2 summarizes the results obtained in the multivariant analysis of the effect biomarkers, taking into account lifestyle factors, including age, gender, and smoking habits, in addition to exposure. It should be noted that only significant results are shown. The significant effect of exposure was confirmed in the MN test and SCE as increases and in %CD19$^+$ B lymphocytes as a decrease. Further, a significant correlation was obtained between frequencies of MN and SCE, and a nearly significant correlation was found between SCE and %CD19$^+$. A significant effect of age was observed on SCE frequency; a rise was also detected for MN rate that did not reach significance. Indeed, a significant positive correlation was found between age and SCE frequency. No significant influence of gender or smoking habits was observed. Besides, exposure time (years of employment) was not significantly correlated with any genotoxicity or immunological parameters.

When considering the FA environmental levels corresponding to each exposed individual, a significant influence of the FA level of exposure was found for some lymphocyte subpopulations based upon significant results collected in Table 3. Total %CD3$^+$ T lymphocytes and %CD4$^+$ Th cells was significantly elevated with FA-level of exposure, whereas %CD16-56$^+$ NK cells was significantly reduced. In fact, these results agree with the correlations found between the FA-exposure levels and %CD3$^+$, %CD4$^+$, and %CD16-56$^+$ NK cells. Significant correlations were also observed between %CD16-56$^+$ NK cells and %CD3+ T lymphocytes and %CD4+ Th cells. No significant effect of gender, smoking habits, or age was noted in these studies.

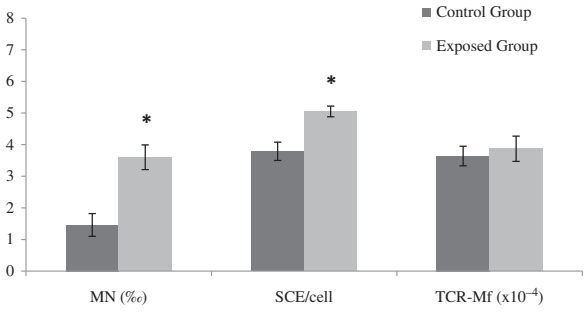


FIGURE 1. Results of MN test, SCE, and TCR mutation assay in the study population. Asterisk indicates significantly different from control group, $p < .05$, according to Student's *t*-test.

DISCUSSION

In pathology anatomy labs, FA is a well-known compound traditionally used as a fixative and tissue preservative. Indoor air analyses consistently show that the levels of airborne FA in pathology anatomy labs exceeded recommended exposure criteria (Shaham et al., 2002; Akbar-Khanzadeh and Pulido, 2003). In these settings, absorption of FA occurs mainly through inhalation. Inhaled FA primarily affects the upper airways; the severity and extent of physiological response depend upon chemical concentration in the air. In the current study the mean FA level of exposure found, 0.36 ± 0.03 ppm, was higher than the established

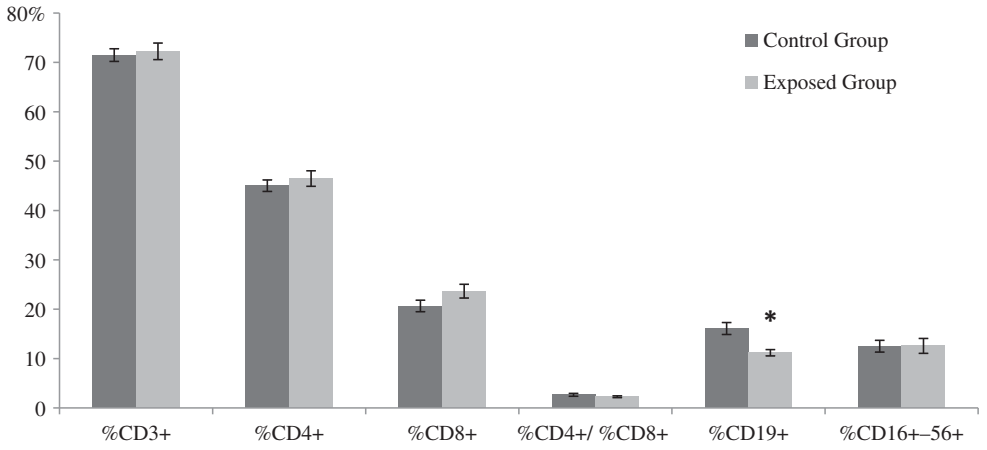


FIGURE 2. Percentages of lymphocyte subsets analyzed in the study population. Asterisk indicates significantly different from control group, $p < .05$, according to Student's *t*-test.

TABLE 2. Influence of Exposure, Gender, Smoking Habits, and Age on MN, SCE, and B Lymphocytes (%CD19$^+$)

Model	Unstandardized coefficients β	95% CI	Partial *p* value	R^2	Model *p* value
1. MN (‰)				.232	**.002**
Exposed vs. nonexposed	2.1	1.025 to 3.174	**<.001**		
Females vs. males	0.334	1.170 to 1.838	.659		
Smokers vs. nonsmokers	0.061	−1.302 to 1.423	.929		
Age (yr)	0.05	−0.008 to 0.109	.088		
2. SCE/cell				.250	**.001**
Exposed vs. nonexposed	1.245	0.594 to 1.897	**<.001**		
Females vs. males	0.514	−0.398 to 1.426	.264		
Smokers vs. nonsmokers	0.28	−0.546 to 1.106	.501		
Age (yr)	0.036	0.001 to 0.071	**.045**		
3. B lymphocytes (%CD19$^+$)				.174	**.014**
Exposed vs. nonexposed	−1.387	−1.714 to − 1.121	**.003**		
Females vs. males	−1.097	−1.477 to 1.228	.536		
Smokers vs. nonsmokers	1.198	−1.093 to 1.568	.185		
Age (yr)	−1.007	−1.019 to 1.004	.228		

$p < 0.05$, significantly different.

national ceiling limit of 0.3 ppm. It is important to note that 54% of the workers were exposed to FA levels greater than or equal to 0.3 ppm, and half of these were exposed to concentrations above 0.4 ppm. Therefore, data obtained show that workers are exposed to levels of FA that are greater than both national and international recommended limit values (IARC, 2006), indicating a potential risk to workers' health. The main FA vapor emissions occurred during macroscopic examination of FA-preserved specimens and during the disposal of specimens and waste solutions. In most cases during these tasks the workers were only using masks for biological hazard, not appropriate to protect from FA vapors. The primary reason given by workers for not using goggles and appropriate masks (when available) was interference in efficiency in performance of activities, namely, difficulties in communication, taking notes, and handling material. Implementation of security and hygiene measures, such as periodic air sampling and medical surveillance, as well as good practice campaigns may be crucial to lower the risk associated with FA occupational exposure.

As shown in Figure 1, frequencies of MN and SCE were significantly elevated in FA-exposed individuals compared to unexposed controls. The increase in MN formation in

TABLE 3. Influence of FA Environmental Level, Gender, Smoking Habits, and Age on T Lymphocytes (%CD3+), T-Helper Cells (%CD4+), and NK Cells (%CD16-56$^+$), Only in the Exposed Population

Model	95% CI	Partial p value	R^2	Model p value
1. Lymphocytes T (%CD3$^+$)			.303	**.024**
FA level of exposure	14.092 to 55.191	**.002**		
Females vs. males	−4.570 to 11.131	.400		
Smokers vs. nonsmokers	−9.623 to 5.928	.631		
Age (yr)	−0.371 to 0.380	.981		
2. T-helper cells (%CD4$^+$)			.280	**.037**
FA level of exposure	8.907 to 47.963	**.006**		
Females vs. males	−4.366 to 10.554	.404		
Smokers vs. nonsmokers	−2.009 to 12.769	.147		
Age (yr)	−0.429 to 0.285	.682		
3. NK cells (%CD16-56$^+$)			.500	<**.001**
FA level of exposure	−98.593 to − 6.821	<**.001**		
Females vs. males	−2.604 to 1.066	.084		
Smokers vs. nonsmokers	−1.255 to 2.190	.270		
Age (yr)	−1.027 to 1.022	.820		

$p < 0.05$, significantly different.

individuals occupationally exposed to FA is well established. Suruda et al. (1993) found elevated frequencies of MN in lymphocytes (26%), nasal epithelial cells (22%), and buccal mucosa cells (12-fold) in a group of mortician students, before and after attending an embalming course. A dose-response relationship was observed with cumulative exposure to FA. In a population of 151 workers exposed to FA from two plywood factories, Yu et al. (2005) reported a significantly higher frequency of MN in peripheral lymphocytes. Further, a higher frequency of MN in lymphocytes was observed in pathology anatomy lab workers in two independent Portuguese studies (Costa et al., 2008; Viegas et al., 2010). Orsière et al. (2006) found a significantly higher frequency of monocentromeric MN in FA-exposed workers, suggesting an aneugenic effect of FA. However, recent studies by Costa et al. (2011) of FA-exposed pathology anatomy workers and by Speit et al. (2011) in mammalian cell lines indicated a clastogenic effect attributed to FA as the primary mechanism underlying MN formation, confirming previous reports (Titenko-Holland et al., 1996).

Our results on SCE frequency also agree with data from other studies that also reported a higher frequency of this cytogenetic biomarker in FA exposed workers. Yager et al. (1986) were the first to describe a rise in SCE in the peripheral lymphocytes of FA-exposed individuals. SCE were measured in blood samples collected from 8 nonsmoking anatomy students before and after a 10-wk anatomy class with a mean FA concentration in breathing-zone samples of 1.2 ppm. The results showed that SCE frequency was significantly higher in samples taken at the end of the course compared to samples obtained from the same individuals immediately before FA exposure began. Shaham et al. (1997, 2002) examined a group of 90 pathology workers. Based on different FA exposure levels, the exposed group was divided into a low-exposure group (0.04 to 0.7 ppm) and a high-exposure group (0.72 to 5.6 ppm). Both exposed groups displayed significantly higher SCE frequencies. Ye et al. (2005) examined two different groups of FA-exposed workers. One group was composed of 18 workers in a FA manufacturing facility (8-h TWA level of exposure was 0.82 ± 0.24 ppm), whereas the second group included 16 waiters who were exposed to low levels of FA while working in a new ballroom for 12 wk (5-h TWA level of exposure was 0.09 ± 0.05 ppm). The first group showed a significantly elevated frequency of SCE in lymphocytes, whereas no significant difference was found in waiters, probably due to the low level of exposure to FA. Finally, Costa

et al. (2008) in a group of pathology anatomy workers (TWA = 0.44 ppm) also noted a 1.4-fold increase in SCE frequency. However, other investigators did not find greater SCE rates in subjects occupationally exposed to FA (Thomson et al., 1984; Suruda et al., 1993; Ying et al., 1999). In the current study no significant differences were found in TCR-Mf. To our knowledge this is the first report in which TCR-Mf was evaluated in workers exposed to FA. Thus, more studies are needed to confirm these findings.

The immunological status of exposed and control subjects was also addressed by assessing percent of different lymphocyte subsets in peripheral blood. The reference values for lymphocyte subsets in Caucasian population are 60–87% for total $CD3^+$ T lymphocytes, 32–61% for $CD4^+$ T helper cells, 14–43% for $CD8^+$ T cytotoxic cells, 5–20% for $CD19^+$ B cells, and 4–28% for NK cells ($CD16^+$-56^+) (Santagostino et al., 2009). Our results in both groups are within these expected ranges and also in accordance with recent data in a Portuguese population (García-Lestón et al., 2011).

Regarding the FA-exposure effect when comparing both groups, no statistical differences were found for total %$CD3^+$ T lymphocytes, %$CD4^+$ T helper cells (Th), %$CD8^+$ T cytotoxic cells (Tc), Th/Tc ratio, and %CD16-56^+ NK cells. However a significant decrease of percent B cells was found in the exposed group. In addition, a nearly significant correlation obtained between SCE and B cells indicates a reliable association between these two parameters influenced by FA exposure.

Our results are in agreement with other studies which showed that FA exposure may affect immunological parameters. Tang et al. (2009) summarized eight Chinese studies concerning FA-induced hematotoxicity. The majority of these reports noted a decrease in total white blood cells (WBC) counts in FA-exposed workers. In one of the studies concerning clinical pathology personnel (FA levels were between 0.2 and 0.8 ppm). Tang et al. (2009) found that a significantly higher proportion of the exposed subjects (14%) showed abnormal WBC counts compared to controls (5%). In a recent study on the ability of FA to disrupt hematopoiesis in a group of exposed workers with mean FA 8-h TWA of 1.28 ppm, Zhang et al. (2010) also reported a significant reduction in WBC counts, granulocytes, and lymphocytes. However, there are also studies where WBC counts were not markedly influenced by FA exposure (Madison et al., 1991; Tang et al., 2009).

In our study a decreased percentage of B cells was found in exposed individuals, suggesting immunosuppression and therefore a reduced immune response. B cells are involved in organism humoral responses against antigens and are also engaged in adaptive immune responses. Further, %CD16-56^+ NK cells were inversely correlated with FA levels of exposure. NK cells are effector lymphocytes of the innate immune system that control several types of tumors and microbial infections by limiting their spread and subsequent tissue damage (Vivier et al., 2009). Indeed, several studies describe a reduced resistance to infections, including upper respiratory tract infections, recurrent rhinitis, and pneumonitis in individuals occupationally exposed to FA. In the study by Zhang et al. (2010), 40% of the exposed subjects had recent respiratory infections. Evidence suggests that FA exposure may result in functional changes in neutrophils and possibly influence the host capacity to respond to infections (Lyapina et al., 2004). Lyapina et al. (2004) tested this hypothesis by measuring neutrophil respiratory burst activity (NRBA) in 29 workers exposed to FA (mean FA level 0.71 $\pm$ 0.32 ppm). Exposed workers displayed a significant rise in upper respiratory tract inflammations, but no significant differences were found between groups in spontaneous or stimulated NRBA assays.

In a population-based case-control study conducted by Hildesheim et al. (2001), the risk of nasopharyngeal carcinoma and ever exposure to FA was higher among Epstein–Barr virus (EBV) seropositive individuals than among nonseropositive subjects. Viral-associated cancers are increased in immunosuppressed individuals, due most likely to the inability of

the organism to limit viral replication and/or expansion of infected cells (Schulz, 2009). Epstein–Barr virus (EBV) is implicated in the etiology of nasopharyngeal carcinoma (Marsh et al., 2007; Bosetti et al., 2008). Thompson and Grafstrom (2009) suggested that FA may exert an indirect influence in EBV reactivation through deregulation of nitrosothiol homeostasis and may also interact with the virus to promote epithelial cell transformation.

Although our results are in agreement with published data, lymphocyte subpopulations were affected differently in some other studies. Ying et al. (1999) assessed the lymphocyte subsets in 23 nonsmoking medical students exposed to FA during an 8-wk anatomy lab course (8-h TWA= 0.413 ± 0.243 ppm) and found a significant increase in B cells and a significant fall in total T cells, T-helper-inducer cells, and T-cytotoxic-suppressor cells at the end of the study. A similar result was reported by Ye et al. (2005) for a group of workers from a FA manufacturing facility. In a recent cross-sectional study conducted in China, a decreased in NK cells, regulatory T cells, and CD8 effector memory T cells was reported among FA-exposed workers (Hosgood et al., 2012). The small sample size or/and higher FA level of exposure may have contributed to the different outcomes observed between these and the present study.

Another important factor that may also explain the different results is individual susceptibility. One of the enzymes involved in FA detoxification is the mitochondrial aldehyde dehydrogenase-2 (ALDH2) (Teng et al., 2001). *ALDH2* gene contains an inactive *ALDH2*2* allele; the presence of the mutant allele leads to a decrease or absence of ALDH2 catalytic activity (Brennan et al., 2004). Approximately 50% of East Asians carry the mutant inactive *ALDH2*2* null allele (Goedde et al., 1992; Oota et al., 2004), whereas nearly all Caucasians carry the functional *ALDH2*1/1* genotype (Chambers et al., 2002; Brennan et al., 2004). Thus, larger human studies combining genotoxic, immunological parameters and susceptibility biomarkers are needed in order to understand the potential relationship between FA exposure and these endpoints.

Finally, significant associations were found between FA level of exposure and total %$CD3^+$ T lymphocytes, %$CD4^+$ Th cells, and %CD16-56^+ NK cells in exposed individuals, indicating a reliable relationship between level of exposure and alterations in these immunological markers. Regarding the influence of lifestyle factors and exposure time on the endpoints studied, in this study no significant influence of gender, smoking habits, or years of employment was observed. Age was the only confounder that showed a significant influence but only with respect to SCE frequency. This effect was confirmed with the significant positive correlation noted between age and SCE frequency. Our data agree with previous studies (Kirsch-Volders et al., 2006; Teixeira et al., 2010) reporting a positive association between age and cytogenetic biomarkers related to a progressive increase in spontaneous chromosome instability and loss of efficiency in DNA repair mechanisms, which may result in accumulation of genetic lesions with increasing age (Bolognesi et al., 1999).

CONCLUSIONS

Data obtained in this study indicate that increased genotoxic risk due to FA occupational exposure cannot be excluded. MN and SCE frequencies were significantly elevated in peripheral lymphocytes of pathology anatomy workers exposed to FA. A significant decrease of percentage of B cells in exposed group was also found. Further, a significant relationships between FA level of exposure and increases in %total T lymphocytes and %Th-cells and fall in %NK cells were noted in exposed individuals, indicating that FA exposure may influence immunological parameters. However, these results need to be interpreted with caution, owing to the relatively low number of exposed and control individuals included in this study.

REFERENCES

ACGIH, 2008. *TLV's and BEI's—Based on the documentation of the threshold limit values*

for chemical substances and physical agents & biological exposure indices. Cincinnati, OH: American Conference of Governmental Industrial Hygienists.

Akbar-Khanzadeh, F., and Pulido, E. V. 2003. Using respirators and goggles to control exposure to air pollutants in an anatomy laboratory. *Am. J. Ind. Med*. 43: 326–331.

Akiyama, M., Umeki, S., Kusunoki, Y., Kyoizumi, S., Nakamura, N., and Mori, T. 1995. Somatic-cell mutations as a possible predictor of cancer risk. *Health Phys*. 68: 643–649.

Bolognesi C., Lando C., Forni A., Landini E., Scarpato R., Migliore L., and Bonassi S. 1999. Chromosomal damage and ageing: Effect on micronuclei frequency in peripheral blood lymphocytes. *Age Ageing*. 28: 393–397.

Bonassi, S., Ugolini, D., Kirsch-Volders, M., Strömberg, U., Vermeulen, R., and Tucker, J. D. 2005. Human population studies with cytogenetic biomarkers: Review of the literature and future prospectives. *Environ. Mol. Mutagen*. 45: 258–270.

Bosetti, C., McLaughlin, J. K., Tarone, R. E., Pira, E., and La Vecchia, C. 2008. Formaldehyde and cancer risk: A quantitative review of cohort studies through 2006. *Ann Oncol* 19: 29–43.

Brennan, P., Lewis S., Hashibe, M., Bell, D. A., Boffetta, P., Bouchardy, C., Caporaso, N., Chen, C., Coutelle, C., Diehl S. R., Hayes, R. B., Olshan, A. F., Schwartz, S. M., Sturgis, E. M., Wei Q., Zavras, A. I., and Benhamou, S. 2004. Pooled analysis of alcohol dehydrogenase genotypes and head and neck cancer: A HuGE review. *Am. J. Epidemiol*. 159: 1–16.

Carrano, A., and Natarajan, A., 1988. Considerations for population monitoring using cytogenetic techniques. *Mutat. Res. Human Monitoring (special issue)* 204: 379–406.

Chambers, G. K., Marshall, S. J., Robinson, G. M., Maguire, S., Newton-Howes, J., and Chong, N. L. 2002. The genetics of alcoholism in Polynesians: Alcohol and aldehyde dehydrogenase genotypes in young men. *Alcohol Clin. Exp. Res*. 26: 949–55.

Checkoway, H., Boffetta, P., Mundt, D. J., and Mundt, K. A. 2012. Critical review and synthesis of the epidemiologic evidence on formaldehyde exposure and risk of leukemia and other lymphohematopoietic malignancies. *Cancer Causes Control*. doi:10.1007/s10552-012-0055-2.

Costa, S., Coelho, P., Costa, C., Silva, S., Mayan, O., Santos, L. S., Gaspar, J., and Teixeira, J. P. 2008. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. *Toxicology* 252: 40–48.

Costa, S., Pina, C., Coelho, P., Costa, C., Silva, S., Porto, B., Laffon, B., and Teixeira, J. P. 2011. Occupational exposure to formaldehyde: genotoxic risk evaluation by comet assay and micronucleus test using human peripheral lymphocytes. *J Toxicol Environ Health A* 74: 1040–1051.

Fenech, M., 1993. The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. *Environ. Health Perspect*. 101: 101–107.

Fenech, M. 2007. Cytokinesis-block micronucleus cytome assay. *Nat. Protocol* 2: 1084–1104.

García-Lestón, J., Roma-Torres, J., Vilares, M., Pinto, R., Cunha, L. M., Prista, J., Teixeira, J. P., Mayan, O., Pásaro, E., Méndez, J., and Laffon, B. 2011. Biomonitoring of a population of Portuguese workers exposed to lead. *Mutat. Res*.18: 81–88.

Goedde, H. W., Agarwall, D. P., Fritze, G., Meier-Tackmann, D., Singh, S., Beckmann, G., Bhatia, K., Chen, L. Z., Fang, B., and Lisker, R. 1992. Distribution of *ADH2* and *ALDH2* genotypes in different populations. *Human Genet*. 88: 344–346.

Hildesheim, A., Dosemeci, M., Chan, C. C., Chen, C. J., Cheng, Y. J., Hsu, M. M., Chen, I. H., Mittl, B. F., Sun, B., Levine, P. H., Chen, J. Y., Brinton, L. A., and Yang, C. S. 2001. Occupational exposure to wood, formaldehyde, and solvents and risk of nasopharyngeal carcinoma. *Cancer Epidemiol. Biomarkers Prev*. 10: 1145–1153.

Hosgood, H. D. 3rd, Zhang, L., Tang, X., Vermeulen, R., Hao, Z., Shen, M., Qiu, C.,

Ge, Y., Hua, M., Ji, Z., Li, S., Xiong, J., Reiss, B., Liu, S., Xin, K. X., Azuma, M., Xie, Y., Freeman, L. B., Ruan, X., Guo, W., Galvan, N., Blair, A., Li, L., Huang, H., Smith, M. T., Rothman, N., and Lan, Q. 2012. Occupational exposure to formaldehyde and alterations in lymphocyte subsets. *Am. J. Ind. Med*. doi:10.1002/ajim.22088.

IARC. 2006. Formaldehyde, 2-butoxyethanol and 1-*tert*-butoxy-2-propanol. *IARC Monogr. Eval. Carcinogen. Risks Hum*. 88. Lyon, France: World Health Organization.

Kirsch-Volders, M., Mateuca, R. A., Roelants, M., Tremp, A., Zeiger, E., Bonassi, S., Holland, N., Chang, W. P., Aka, P. V., Deboeck, M., Godderis, L., Haufroid, V., Ishikawa, H., Laffon, B., Marcos, R., Migliore, L., Norppa, H., Teixeira, J. P., Zijno, A., and Fenech, M. 2006. The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo. *Cancer Epidemiol. Biomarkers Prev*. 15: 1038–1042.

Laffon, B., Teixeira, J. P., Silva, S., Roma-Torres, J., Pérez-Cadahía, B., Méndez, J., Pásaro, E., and Mayan, O. 2006. Assessment of occupational genotoxic risk in the production of rubber tyres. *Ann. Occup. Hyg*. 50: 583–592.

Lu, K., Boysen, G., Gao, L., Collins, L. B., and Swenberg, J. A. 2008. Formaldehyde-induced histone modifications in vitro. *Chem. Res. Toxicol*. 21: 1586–1593.

Luebke, R. W., Chen, D. H., Dietert, R., Yang, Y., and Luster, M. I., with Immunotoxicology Workshop. 2006. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J. Toxicol. Environ. Health B* 9: 1–26.

Lyapina, M., Zhelezova, G., Petrova, E., and Boev, M. 2004. Flow cytometric determination of neutrophil respiratory burst activity in workers exposed to formaldehyde. *Int. Arch. Occup. Environ. Health* 77: 335–340.

Madison, R. E., Broughton, A., and Thrasher, J. D. 1991. Immunologic biomarkers associated with an acute exposure to exothermic byproducts of a urea formaldehyde spill. *Environ. Health Perspect*. 94: 219–223.

Marsh, G. M., Youk, A. O., and Morfeld, P. 2007. Mis-specified and non-robust mortality risk models for nasopharyngeal cancer in the National Cancer Institute formaldehyde worker cohort study. *Regul. Toxicol. Pharmacol*. 47: 59–67.

Mayeux., R. 2004. Biomarkers: Potential uses and limitations. *NeuroRx* 1: 182–188.

National Toxicology Program. 2010. Final report on carcinogens background document for formaldehyde. *Rep. Carcinogen Background Doc*. 10-5981:i-512.

National Institute for Occupational Safety and Health. 1994. Formaldehyde: Method 3500 (Issue 2). In *NIOSH manual of analytical methods*, 2–5. Cincinnati, OH: U.S. Department of Health and Human Services.

Oota, H., Pakstis, A. J., Bonne-Tamir, B., Goldman, D., Grigorenko, E., Kajuna, S .L., Karoma, N. J., Kungulilo, S., Lu, R. B., Odunsi, K., Okonofua, F., Zhukova, O. V., Kidd, J. R., and Kidd, K. K. 2004. The evolution and population genetics of the ALDH2 locus: Random genetic drift, selection, and low levels of recombination. *Ann. Hum. Genet*. 68: 93–109.

Orsière, T., Sari-Minodier, I., Iarmarcovai, G., and Botta, A. 2006. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. *Mutat. Res*. 605: 30–41.

Paustenbach, D., Alarie, Y., Kulle, T., Schachter, N., Smith, R., Swenberg, J., Witschi, P., and Horowitz, S. B., 1997. A recommended occupational limit for formaldehyde based on irritation. *J. Toxicol. Environ. Health* 50: 217–263.

Perry, P., and Wolff, S., 1974. New Giemsa method for the differential staining of sister chromatids. *Nature* 251: 156–158.

Santagostino, A., Garbaccio, G., Pistorio, A., Bolis, V., Camisasca, G., Pagliaro, P., and Girotto, M. 1999. An Italian national multicenter study for the definition of reference ranges for normal values of peripheral blood lymphocyte subsets in healthy adults. *Haematologica* 84: 499–504.

Schulz, T. F. 2009. Cancer and viral infections in immunocompromised individuals. *Int. J. Cancer* 15: 1755–1763.

Shaham, J., Bomstein, Y., Meltzer, A., and Ribak, J. 1997. DNA-protein crosslinks and sister chromatid exchanges as biomarkers of exposure to formaldehyde. *Int. J. Occup. Environ. Health* 3: 95–104.

Shaham, J., Gurvich, R., and Kaufman, Z. 2002. Sister chromatid exchanges in pathology staff occupationally exposed to formaldehyde. *Mutat. Res*. 514: 115–123.

Speit, G., Kahner, S., Linsenmeyer, R., and Schatz, P. 2011. Does formaldehyde induce aneuploidy? *Mutagenesis* 26: 805–811.

Suruda, A., Schulte, P., Boeniger, M., Hayes, R. B., Livingston, G. K., and Steenland, K., 1993. Cytogenetic effects of formaldehyde exposure in students of mortuary science. *Cancer Epidemiol. Biomarkers Prev*. 2: 453–460.

Tang, X., Bai, Y., Duong, A., Smith, M.T., Li, L., and Zhang, L. 2009. Formaldehyde in China: Production, consumption, exposure levels, and health effects. *Environ. Int*. 35: 1210–1224.

Teixeira, J., Gaspar, J., Silva, S., Torres, J., Silva, S., Azevedo, M., Neves, P., Laffon, B., Mendez, J., Goncalves, C., Mayan, O., Farmer, P., and Rueff, J. 2004. Occupational exposure to styrene: modulation of cytogenetic damage and levels of urinary metabolites of styrene by polymorphisms in genes CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1. *Toxicology* 195: 231–242.

Teixeira, J. P., Gaspar, J., Coelho, P., Costa, C., Pinho-Silva, S., Costa, S., Da Silva, S., Laffon, B., Pásaro, E., Rueff, J., and Farmer, P. 2010. Cytogenetic and DNA damage on workers exposed to styrene. *Mutagenesis* 25: 617–621.

Teng, S., Beard, K., Pourahmad, J., Moridani, M., Easson, E., Poon, R., and O'Brian, P. J. 2001. The formaldehyde metabolic detoxification enzyme systems and molecular cytotoxic mechanism in isolated rat hepatocytes. *Chem. Biol. Interact*. 130–132: 285–296.

Thompson, C., and Grafstrom, R. 2009. Commentary: mechanistic considerations for associations between formaldehyde exposure and nasopharyngeal carcinoma. *Environ. Health* 8: 53.

Thomson, E. J., Shackleton, S., and Harrington, J. M. 1984. Chromosome aberrations and sister-chromatid exchanges frequencies in pathology staff occupationally exposed to formaldehyde. *Mutat. Res*. 141: 89–93.

Titenko-Holland, N., Levine, A. J., Smith, M. T., Quintana, P. J., Boeniger, M., Hayes, R., Suruda, A., and Schulte, P. 1996. Quantification of epithelial cell micronuclei by fluorescence in situ hybridization (FISH) in mortuary science students exposed to formaldehyde. *Mutat. Res*. 371: 237–248.

Veraldi, A., Costantini, A. S., Bolejack, V., Miligi, L., Vineis, P., and van Loveren, H. 2006. Immunotoxic effects of chemicals: A matrix for occupational and environmental epidemiological studies. *Am. J. Ind. Med*. 49: 1046–1055.

Viegas, S., Ladeira, C., Nunes, C., Malta-Vacas, J., Gomes, M., Brito, M., Mendonca, P., and Prista, J. 2010. Genotoxic effects in occupational exposure to formaldehyde: A study in anatomy and pathology laboratories and formaldehyde-resins production. *J. Occup. Med. Toxicol*. 5: 25.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. 2009. Functions of natural killer cells. *Nat. Immunol*. 9: 503–510.

Yager, J. W., Cohn, K. L., Spear, R. C., Fisher, J. M., and Morse, L., 1986. Sister-chromatid exchanges in lymphocytes of anatomy students exposed to formaldehyde-embalming solution. *Mutat. Res*. 174: 135–139.

Ye, X., Yan, W., Xie, H., Zhao, M., and Ying, C., 2005. Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. *Mutat. Res*. 588: 22–27.

Ying, C. J, Ye, X. L., Xie, H., Yan, W. S., Zhao, M. Y., Xia, T., and Yin, S. Y. 1999. Lymphocyte subsets and sister-chromatid exchanges in the students exposed to formaldehyde vapor. *Biomed. Environ. Sci*. 12: 88–94.

Yu, L. Q., Jiang, S. F., Leng, S. G., He, F. S., and Zheng, Y. X. 2005. Early genetic effects on workers occupationally exposed to formaldehyde. *Chin. J. Prev. Med*. 39: 392–395.

Zhang, L., Freeman, L. E., Nakamura, J., Hecht, S. S., Vandenberg, J. J., Smith, M. T., and Sonawane, B. R. 2010. Formaldehyde and leukemia: Epidemiology, potential mechanisms, and implications for risk assessment. *Environ. Mol. Mutagen*. 51: 181–191.

Micronucleus frequencies in lymphocytes and buccal cells in formaldehyde exposed workers

S. Costa[1], F. Brandão[1,2], M. Coelho[1], C. Costa[1,3], P. Coelho[1,2], S. Silva[1], B. Porto[4] & J. P. Teixeira[1,3]
[1]*National Institute of Health, Environmental Health Department, Portugal*
[2]*Institute of Agricultural and Agri-foodstuffs Sciences and Technologies (ICETA), University of Porto, Portugal*
[3]*Institute of Public Health, University of Porto (ISPUP), Portugal*
[4]*Institute of Biomedical Sciences Abel Salazar (ICBAS), Laboratory of Cytogenetics, University of Porto, Portugal*

Abstract

Formaldehyde (FA) is a high-volume production chemical produced worldwide with a large range of industrial and medical uses. Listed, since 2004, by IARC as a human carcinogen, FA status was recently revised by the US government who reclassified this compound as known to be a human carcinogen. Both reclassifications are based on sufficient evidence of carcinogenicity from epidemiologic studies, supporting data on mechanisms of carcinogenesis and experimental evidence in animals. The highest level of human exposure to FA occurs in occupational settings. Consistent findings of increased risks of certain types of rare cancers were found among workers with higher measures of exposure to FA (exposure level or duration). The aim of the present study was to assess the genotoxic effects of occupational exposure to FA. A group of pathology and anatomy workers was evaluated for micronuclei in lymphocytes and in exfoliated buccal cells. Genotoxic endpoints are of great interest in the risk assessment of occupational carcinogens because they precede by a long time the potential health effects, thus offering a greater potential for preventive measures. Micronuclei in lymphocytes and in exfoliated buccal cells were significantly higher in the exposed subjects when compared with controls. Air sampling was performed in the workers' breathing zone for representative working periods and an 8h-time weighted average was assessed. Results

WIT Transactions on Biomedicine and Health, Vol 16, © 2013 WIT Press
www.witpress.com, ISSN 1743-3525 (on-line)
doi:10.2495/EHR130081

obtained confirm an association between genetic damage and occupational exposure to FA. Such results along with the recent implications of human carcinogenicity, point out the need for close monitoring of FA exposures. Implementation of effective control measures along with hazard prevention campaigns may be crucial to decrease the risk.
Keywords: formaldehyde, genotoxicity, micronucleus test, lymphocytes, exfoliated buccal cells.

1 Introduction

Over the years several epidemiological studies have revealed an increased risk of cancer development among workers exposed to chemicals [1]. Human monitoring is a frequently used approach to provide early warning signals for excessive exposure to toxic substances and for prediction of health risk [2].

Establishing the health outcomes of various activities and exposures requires information about the levels of exposure and the biological effects resulting from the interaction between the exposed organism and the chemical agent. Genomic damage is probably the primary basic cause of developmental and degenerative disease. It is well established that genomic damage may result from lifestyle factors, medical procedures (e.g., chemotherapy, radiotherapy), diet, individual susceptibility and environmental/occupational exposure to genotoxic compounds.

A wide range of bioindicators are currently used for the detection of early biological effects of genotoxic agents, namely cytogenetic alterations. The relevance of increased frequency of cytogenetic alterations as a cancer risk biomarker is further supported by epidemiological studies suggesting that a high frequency of chromosomal aberrations or micronucleus are predictive of an increase risk of cancer [3].

Micronucleus (MN) test is a sensitive and well established tool extensively used in human biomonitoring studies to assess DNA damage at chromosomal level [4]. Micronuclei are extra-nuclear DNA containing bodies formed as a result of chromosomal fragments or whole chromosomes not being incorporated into the daughter nuclei during nuclear division. Since MN represents a measure of both chromosome breakage and chromosome loss, an increased frequency of micronucleated cells can reflect exposure to genotoxic agents with clastogenic or aneugenic modes of action. In human studies, peripheral blood lymphocytes are usually the most frequently used tissue for MN test. However exfoliated epithelial cells (urothelial, buccal or nasal cells) are increasingly popular, as they can be easily collected and in some cases are better models than lymphocytes, since they are target tissues of some cancers.

Formaldehyde (FA) is a building-block for many chemical compounds with a wide range of industrial and medical uses. It is a high-volume production chemical produced worldwide, which many people are exposed to. At room temperature it is a flammable and colorless gas with a strong pungent odor. In 2006, the International Agency for Research on Cancer, IARC, reclassified FA from Group 2A (probably carcinogenic to humans) to Group 1 (carcinogenic to

humans) [5]. More recently, FA carcinogenic status was also revised by the US National Toxicology Programme (NTP). After a rigorous scientific review, FA was reassigned, in the NTP 12th Report on Carcinogens, as *known to be a human carcinogen* [6]. Both reclassifications are based on sufficient evidence of carcinogenicity from studies in humans, supporting studies on mechanisms of carcinogenesis and experimental evidence in animals.

Epidemiological studies demonstrated a causal relationship between occupational exposure to FA and cancer [5, 6]. Consisting findings of increased risks of certain types of rare cancers, namely nasopharyngeal and sinonasal carcinomas were found among workers with higher measures of exposure to FA (exposure level or duration). Studies have also suggested that FA may affect the lymphatic and blood systems and that exposure to FA may cause leukemia, particularly myeloid leukemia, in humans [7–9], yet due to mix results and biological implausibility the evidence for FA leukemogenicity remains controversial and needs further investigation [5, 10]. FA also caused tumors in two rodent species (rats and mice), at several different tissue sites, and by two different routes of exposure (inhalation and ingestion) [6].

The highest level of human exposure to FA occurs in occupational settings, namely in pathology and anatomy laboratories where it is used as a fixative and tissue preservative for more than 100 years. Indoor air analyses have consistently shown that the levels of airborne FA in anatomy laboratories exceed recommended exposure criteria [11, 12]. In these settings, absorption of FA occurs mainly through inhalation, affecting primarily the upper airways.

In the last decade a large number of toxicological studies were published about FA. FA's genotoxicity is confirmed in a variety of experimental systems ranging from bacteria to rodents. Although these positive findings may provide a basis for extrapolation to humans, the cytogenetic assays in humans have been inconsistent with both positive and negative outcomes [5]. Biological evidence of toxicity on distant-site such as peripheral lymphocytes and bone marrow is still insufficient and conflicting [13]. Some authors stated that since inhaled FA is rapidly metabolized it would not be expected to enter the systemic circulation and for that reason genotoxic and carcinogenic effects (leukemia) in animals and humans are limited to local effects, in the area of first contact [14, 15]. The principal aim of the present study was to evaluate MN frequency in both peripheral blood lymphocytes (PBLs) and in exfoliated buccal cells of FA-exposed workers from pathology anatomy laboratories. An association on MN frequency in these two biological tissues, a first contact tissue and a systemic tissue, was also investigated. Air sampling was also performed in order to determine FA-level of exposure of each worker.

2 Methods

2.1 Study population

The general characteristics of the studied population are summarized in Table 1. In total, 80 women were involved in the study, 38 working for at least one year

in pathology and anatomy laboratories located in Portugal, and 42 non-exposed control females, working in administrative offices in the same area and without occupational exposure history to formaldehyde (FA). Both groups were similar in age and smoking habits. Health conditions, medical history, medication, diagnostic tests (X-rays, etc) and important individual information namely age and smoking habits was elicited by means of questionnaires. Subjects that stopped smoking for more than two years were considered non-smokers. Workers also gave information related to working practices such as use of protective measures, years of employment, specific symptoms related to FA-exposure and chronic respiratory diseases and others. Ethical approval for this study was obtained from the Ethical Board of the National Institute of Health. All subjects were fully informed about the procedures and aims of this study and each subject prior to the study signed an informed consent form.

Table 1: Characteristics of the study population.

	Control Group (*N*=42)	**Exposed Group** (*N*=38)	*P*-value
Age (years) [a]	38.90 ± 11.99 (*20–61*)	39.68 ± 8.49 (*26–56*)	*0.74*[b]
Years of employment [a]	——	11.82 ± 7.10 (*1–32*)	
Smoking status			
Non-smokers	34 (81%)	30 (79%)	
Smokers	8 (19%)	8 (21%)	
Packs per year [a]	13.14 ± 9.76 (*2.85–32.25*)	9.07 ± 6.32 (*0.25–18.00*)	*0.34*[b]

[a]Mean ± SD (*range*).
[b]Student's *t*-test.

2.2 Environmental monitoring

Air sampling was performed in the workers breathing zone for representative working periods; analysis of the samples allowed the calculation of the 8h-TWA (time weighed average) level of exposure to FA for each subject. Air sampling and FA analysis were performed according to the NIOSH method no. 3500 [16].

2.3 Biologic samples collection

For lymphocytes culture peripheral blood samples were collected by venipuncture from each donor. After rinsing the mouth with tap water, buccal cells were collected from each donor. This was performed inside of both cheeks with different cytobrush to sample left and right areas of the mouth, to eliminate any unknown bias that may be caused by sampling one cheek only. All samples were collected between 10 and 11 am, coded, and analyzed under blind conditions.

2.4 Lymphocyte cytokinesis-block micronucleus assay

Aliquots of 0.5 mL of whole blood were used to establish duplicate lymphocyte cultures for cytokinesis-blocked micronucleus (MN) test, as described by Teixeira *et al*. [17]. Microscopic analyses were performed on a Nikon Eclipse E400 light microscope. To determine the total number of MN in binucleated cells, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject. MN were scored blindly by the same reader and identified according to the criteria defined by Fenech [18].

2.5 Buccal micronucleus cytome assay

Buccal micronucleus cytome assay was performed as described by Thomas *et al.* [19] with minor modifications.Briefly, for every subject, an individual sample from each cheek was collected and suspended in a 10 mL tube with buccal cell buffer (0.01M Tris-HCl, 0.1M EDTA, 0.02 NaCl, pH7). Cells were then spun for 10 min at 1500 rpm. Supernatant was removed and replaced with fresh buffer and washed twice more. After the last wash, cells were resuspended in small volume of buffer and placed in clean labeled slides (3 slides for each cheek). After air-drying, slides were fixed with cold ethanol: acetic acid (3:1, v/v) solution for 20 min. Air-dried slides were then treated in 5M HCl for 30 min and washed in running tap water for 3 min. Slides were then stained with Schiff's reagent at room temperature, in the dark, for at least 60 min. Next, slides were washed in running tap water for 5 min and 1 min in deionised water and left to dry in the dark for 10 min. Slides were then stained for 5 sec in 1% Fast Green solution and washed in ethanol 3 times, 2min each. After that, slides were allowed to air dry, then covered with coverslips and mounted with Entellan®. Slides were scored blindly by the same person using a Nikon Eclipse E400 light microscope. For each subject a total of 1000 cells were scored for basal cells, differentiated cells, binucleated cells and cell death parameters such as condensed chromatin, karyorrhectic, pyknotic and karyolitic cells. A total of 2000 differentiated cells were scored for micronuclei, nuclear buds and nucleoplasmic bridges. Cells containing micronuclei were confirmed under fluorescence to eliminate false positives. The scoring criteria were based in Tolbert *et al.* [20] and Thomas *et al.* [19].

2.6 Statistical analysis

All analyses were conducted using the SPSS for Windows statistical package 16.0. The statistical differences between means in the characteristics of the study population were assessed by means of students' *t*-test. All results obtained in the study were assessed for normal distribution using Kolmogorov-Smirnov test and graphic evaluation (histograms, Q-Q plots). Since both dependent variables departed from normal distribution non-parametric tests were applied to data. The effect of exposure on the level of genotoxicity was preliminarily tested through Mann-Whitney *U*-test. Multivariant analysis with Negative Binomial model (with log link) was applied to evaluate the contribution of exposure and potential

confounding factors to the response variables considered. Correlation between variables was analyzed by Spearman's correlation test. The level of significance considered was 0.05.

3 Results and discussion

To evaluate current exposure to formaldehyde (FA) in pathology and anatomy laboratories air samples were collected in the worker's breathing zone. The mean level of worker's exposure to FA was 0.35 ± 0.03 ppm (range 0.18–0.69 ppm). Current Portuguese occupational exposure limit is 0.30 ppm (ceiling level), meaning the maximum safe FA concentration that should never be exceeded during any length of time in a worker's breathing zone**.** The American Conference of Governmental Industrial Hygienists (ACGIH) also set a ceiling exposure limit of 0.30 ppm [21]. Our results show that in the laboratories analysed the pathology anatomy workers are exposed to air concentrations of FA that exceed national guidelines and ACGIH recommended exposure criteria. Several reports on indoor FA levels have consistently shown that the airborne concentrations in anatomy laboratories approach or exceed recommended guidelines [11, 12]. Implementation of security and hygiene measures, such as periodic air sampling, efficient air extraction, temperature control, as well as good practice campaigns, may be crucial to decrease FA exposure in this workplace.

The results of MN frequency in peripheral blood lymphocytes (PBLs) and buccal cells are shown in Table 2. Compared to unexposed controls MN frequency was significantly increased in FA-exposed females in both PBLs and exfoliated buccal cells. MN frequency in PBLs was found to be 2.1-times higher in the exposed workers compared to control subjects, whereas in buccal cells a 5.9-fold increase was observed.

Table 2: Results of biomarkers of genotoxicity in studied groups (mean ± SE and range).

	Control Group	**Exposed Group**
PBLs MN (‰)	1.71 ± 0.25 (*0–6*)	3.51 ± 0.35* (*0–8*)
Buccal MN (‰)	0.12 ± 0.06 (*0–2*)	0.71 ± 0.12 * (*0–4*)

S.E., mean standard error.
*P< 0.05.

Our results are in agreement with data obtained in other studies that reported an increased frequency of MN in PBLs and/or epithelial cells among mortuary students and, more recently, in hospital staff exposed to FA. In 1993, Suruda *et al.* [22] reported a significant increase in post-exposure MN frequency in lymphocytes (26%) and buccal mucosa cells (12-fold) among mortician students exposed to FA (TWA=0.33ppm), whereas in nasal epithelial cells the increase

(22%) was not significant. Also, a significant dose-response relationship was found between buccal micronuclei increase and cumulative exposure to FA, but only in male subjects. Ying *et al.* [23] also assessed MN frequency in lymphocytes, oral and nasal mucosa cells of 25 anatomy students exposed to FA over an 8-week period. A higher frequency of MN was found in nasal and oral exfoliated cells, but no significant increase in lymphocytes was observed. In contrast, a significant elevated frequency in micronucleated lymphocytes was found among 59 pathology and anatomy laboratory workers in a study conducted by Orsière *et al.* [24]. Burgaz *et al.* [25, 26] also evaluated MN induction on pathology and anatomy workers exposed to FA but only in buccal and nasal epithelium; an increased frequency of MN in both epithelial cells was found in exposed subjects compared to controls. More recently in a group of Portuguese histopathology laboratory workers (Ladeira *et al.* [27]) reported elevated MN frequencies in PBLs and buccal epithelial cells, confirming previous reports by Costa *et al.* [13, 28] and Viegas *et al.* [29].

Increased frequencies of this biomarker were also found among FA-exposed workers from industrial units. In fact, Ballarin *et al.* [30] were the first to describe an increase in micronucleated nasal cells collected from 15 non-smoking workers from a plywood factory. TWA levels of exposure to FA were 0.08 ppm in the saw mill and shearing-press departments and 0.32 ppm in the warehouse area, there was also a concurrent exposure to wood dust (0.19 to 0.6 ppm). Later on, in a population of 151 plywood factory workers exposed to FA, Yu *et al.* [31] also reported a significantly higher frequency of MN but in PBLs, compared to a control group.

Overall, the majority of the studies show a link between the exposure to this chemical and the increase of this cytogenetic endpoint in lymphocytes, oral and nasal epithelium, confirming that MN is a sensitive indicator for the mutagenic action of FA.

Micronuclei formation may result from aneugenic or clastogenic actions. On additional slides from Suruda *et al.* [22] study, Titenko-Holland *et al.* [32] detected a greater increase in centromere-negative micronuclei content in buccal and nasal tissues from FA-exposed subjects suggesting chromosome breakage as the primary mechanism of FA micronucleus formation. In contrast, Orsière *et al.* [24], in their study found higher significant frequencies of centromere-positive micronuclei (monocentric) in the FA-exposed workers. However, recent studies [28, 33] indicate a clastogenic effect of FA, confirming earlier results from Titenko-Holland *et al*. [32] study.

In the present work we found a significant positive association (r=0.449, P<0.001) between MN frequencies in PBLs and exfoliated buccal cells. The concomitant increase of MN formation in buccal cells, a local FA-target tissue, and in PBLs indicates not only that inhaled FA is able to induce cytogenetic alterations in circulating systemic lymphocytes (distal tissue) but also that the damage between this two tissues may be associated, giving relevance to Zhang *et al.* [9, 34] hypothesis of FA capability to induce directly or indirectly genotoxic damage in distal cells. Although the biological mechanisms associated with FA-induced cancer are not completely understood, it is important to acknowledge

that chemicals can act through multiple toxicity pathways and modes and/or mechanisms of action to induce cancer or other health outcomes [35].

Potential carcinogenic modes of actions for FA include DNA reactivity, gene mutation, chromosomal breakage, aneuploidy, enzyme-mediated DNA damage/repair, cell signaling other than nuclear-receptor mediated, immune response modulation, inflammation, and cytotoxicity [6].

Among the exposed group, was also found a near significant positive association (r=0.324, P=0.051) between MN frequency in PBLs and FA exposure levels, which reinforces the relevance of using this cytogenetic damage biomarker to assess FA genotoxic effects in occupational exposed populations.

The evaluation of the effects of age and smoking habits in addition to exposure was performed using a multivariate model. Table 3 summarizes the results obtained.

Table 3: Influence of exposure, smoking habits and age on MN frequency in PBLs and buccal cells.

Model	β	SE	95% Wald CI	Wald χ^2	P-value
1. PBLs MN (‰)					
Exposed vs. non-exposed	0.72	0.27	0.19 to 1.25	7.20	**<0.05**
Smokers vs. non-smokers	0.03	0.33	-0.62 to 0.67	0.01	0.93
Age (years)	0.01	0.01	-0.01 to 0.04	1.26	0.26
2. Buccal MN (‰)					
Exposed vs. non-exposed	1.92	0.56	0.81 to 3.02	11.54	**<0.05**
Smokers vs. non-smokers	0.85	0.46	-0.06 to 1.75	3.36	0.07
Age (years)	0.04	0.03	-0.01 to 0.09	2.25	0.13

In the present study no significant influence of age, smoking habits or years of employment was observed. A significant positive correlation was found between age and MN frequency in buccal cells (r=0.257, P<0.05). The increase of MN frequency in buccal cells with age is a documented fact [36] confirmed in a recent pooled analysis by Bonassi *et al.* [37] with more than 5000 subjects, being this increase significant from age forty.

In conclusion, results found in the present study substantiate an association between MN formation and occupational exposure to FA, confirming this endpoint as a sensitive indicator for evaluating FA genotoxic effect in occupational exposed populations. Moreover, a significant positive correlation between MN frequency in PBLs and MN frequency in buccal cell was found. Such results along with the recent implications of human carcinogenicity, point out for the need of close monitoring of FA exposures. Development of training programs, medical surveillance programs, valuable data for program evaluation and effective purchase and implementation of control measures may be crucial to decrease the risk associated to FA occupational exposure.

Acknowledgements

This work is supported by Fundaçãopara a Ciência e a Tecnologia (FCT) under the grants SFRH/BD/46929/2008 and PTDC/SAU-ESA/102367/2008.

References

[1] Clapp, R.W., Jacobs, M.M. and Loechler, E.L., Environmental and occupational causes of cancer: new evidence 2005–2007. *Rev Environ Health*, **23(1)**, pp. 1–37, 2008.

[2] Cebulska-Wasilewska, A., Srám, R. J. and Au, W.W., Human Monitoring for Genetic Effects (NATO ASI SERIES), *IOS Press*: Amsterdam, volume 351, 2003.

[3] Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M. and *et al*., An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*, **28(3)**, pp. 625–31, 2007.

[4] Fenech, M., Holland, N., Chang, W. P., Zeiger, E. and Bonassi, S., The Human MicroNucleus Project – An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat. Res.*, 428, pp. 271–28, 1999.

[5] International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risks to Human, Volume 88: Formaldehyde, 2-Butoxyethanol and 1-*tert*-Butoxy-2-propanol. *World Health Organization*: Lyon, 2006.

[6] National Toxicology Program, Final Report on Carcinogens Background Document for Formaldehyde. *Rep Carcinog Backgr Doc*. 10-5981: i-512, 2010.

[7] Bosetti, C., McLaughlin, J.K., Tarone, R.E., Pira, E., and La Vecchia C., Formaldehyde and cancer risk: a quantitative review of cohort studies through 2006. *Ann Oncol,* **19(1)**, pp. 29–43, 2008.

[8] Beane-Freeman L.E., Blair, A., Lubin, J.H., Stewart, P.A., Hayes, R.B., Hoover, R.N. and Hauptmann, M., Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries: the National Cancer Institute Cohort. *J Natl Cancer Inst,* **101(10)**, pp. 751–761, 2009.

[9] Zhang, L., Steinmaus, C., Eastmond, D.A., Xin, X.K. and Smith, M.T. Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms. *Mutat Res,* **681(2–3)**, pp. 150–168, 2009.

[10] Speit, G., Zeller, J., Schmid, O., Elhajouji, A., Ma-Hock. L and Neuss S. Inhalation of formaldehyde does not induce systemic genotoxic effects in rats. *Mutat Res,* **677(1–2)**, pp. 76–85, 2009.

[11] Akbar-Khanzadeh, F. and Pulido, E.V., Using Respirators and Goggles to Control Exposure to Air Pollutants in an Anatomy Laboratory. *Am. J. Ind. Med.*, **43**, pp. 326–331, 2003.

[12] Ohmichi, K., Komiyama, M., Matsuno, Y., Takanashi, Y., Miyamoto, H., Kadota, T., Maekawa, M., Toyama, Y., Tatsugi, Y., Kohno, T., Ohmichi,

M. and Mori, C., Formaldehyde exposure in a gross anatomy laboratory – personal exposure level is higher than indoor concentration. *Environ. Sci. Pollut. Res. Int*., **13**, pp. 120–4, 2006.
[13] Costa, S., Coelho, P., Costa, C., Silva, S., Mayan, O., Santos, L.S, Gaspar, J. and Teixeira, J.P., Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. *Toxicology*, **252(1–3)**, pp. 40–48, 2008.
[14] Heck, H. and Casanova, M., The implausibility of leukemia induction by formaldehyde: a critical review of the biological evidence on distant-site toxicity. *Regul Toxicol Pharmacol* **40(2)**, pp. 92–106, 2004.
[15] Pyatt, D., Natelson, E. and Golden, R. Is inhalation exposure to formaldehyde a biologically plausible cause of lymphohematopoietic malignancies? *Regul Toxicol Pharmacol* **51(1)**, pp. 119–133, 2008.
[16] National Institute for Occupational Safety and Health, Formaldehyde: Method 3500 (Issue 2), in: NIOSH (Eds.), NIOSH Manual of Analytical Method. U.S. Department of Health and Human Services: Cincinnati, Ohio, pp. 2–5, 1994.
[17] Teixeira, J., Gaspar, J., Silva, S., Torres, J., Silva, S., Azevedo, M., Neves, P., Laffon, B., Mendez, J., Goncalves, C., Mayan, O., Farmer, P. and Rueff, J., Occupational exposure to styrene: modulation of cytogenetic damage and levels of urinary metabolites of styrene by polymorphisms in genes CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1. *Toxicology* **195**, pp. 231–42, 2004.
[18] Fenech, M., Cytokinesis-block micronucleus cytome assay. *Nat. Protoc*. **2**, pp. 1084–1104, 2007.
[19] Thomas, P., Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S. and Fenech M., Buccal micronucleus cytome assay. *Nat Protoc.* **4(6)**, pp. 825–37, 2009.
[20] Tolbert, P.E., Shy, C.M. and Allen, J.W., Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat. Res.* **271**, pp. 69–77, 1992.
[21] American Conference of Governmental Industrial Hygienists TLVs and BEIs, Based on the Documentation of the Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices. *American Conference of Governmental Industrial Hygienists*: Cincinnati, 2008.
[22] Suruda, A., Schulte, P., Boeniger, M., Hayes, R.B., Livingston, G.K. and Steenland, K., Cytogenetic effects of formaldehyde exposure in students of mortuary science. *Cancer Epidemiol. Biomarkers Prev.* **2**, pp. 453–460, 1993.
[23] Ying, C.J., Yan, W.S., Zhao, M.Y., Ye, X.L., Xie, H., Yin, S.Y. and Zhu, X.S., Micronuclei in nasal mucosa, oral mucosa and lymphocytes in students exposed to formaldehyde vapor in anatomy class. *Biomed. Environ. Sci.***10**, pp. 451–5, 1997.
[24] Orsière, T., Sari-Minodier, I., Iarmarcovai, G. and Botta, A. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to

formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. *Mutat. Res.* **605 (1–2)**, pp. 30–41, 2006.
[25] Burgaz, S., Cakmak, G., Erdem, O., Yilmaz, M. and Karakaya, A.E., Micronuclei frequencies in exfoliated nasal mucosa cells from pathology and anatomy laboratory workers exposed to formaldehyde. *Neoplasma* **48 (2)**, pp. 144–147, 2001.
[26] Burgaz, S., Erdem, O., Cakmak, G., Erdem, N., Karakaya, A., and Karakaya, A.E., Cytogenetic analysis of buccal cells from shoe-workers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde. *Biomarkers* **7(2)**, pp. 151–61, 2002.
[27] Ladeira, C., Viegas, S., Carolino, E., Prista, J., Gomes, M.C. and Brito, M., Genotoxicity biomarkers in occupational exposure to formaldehyde – the case of histopathology laboratories.*Mutat Res.,* **721(1)**, pp. 15–20, 2011.
[28] Costa, S., Pina, C., Coelho, P., Costa, C., Silva, S., Porto, B., Laffon, B. and Teixeira, J.P., Occupational exposure to formaldehyde: genotoxic risk evaluation by comet assay and micronucleus test using human peripheral lymphocytes. *J Toxicol Environ Health A*. **74(15–16)**, pp. 1040–51, 2011.
[29] Viegas, S., Ladeira, C., Nunes, C., Malta-Vacas, J., Gomes, M., Brito, M., Mendonca, P., Prista, J., Genotoxic effects in occupational exposure to formaldehyde: A study in anatomy and pathology laboratories and formaldehyde-resins production. *J Occup Med Toxicol*. **5(25)**, pp. 1–8, 2010.
[30] Ballarin C, Sarto F, Giacomelli L, Bartolucci GB, Clonfero E., Micronucleated cells in nasal mucosa of formaldehyde-exposed workers. *Mutat Res* **280(1)**, pp. 1–7, 1992.
[31] Yu, L.Q., Jiang, S.F., Leng, S.G., He, F.S., and Zheng, Y.X., Early genetic effects on workers occupationally exposed to formaldehyde. *Chinese Journal of Preventive Medicine* **39**, pp. 392–5, 2005. [abstract].
[32] Titenko-Holland, N., Levine, A.J., Smith, M.T., Quintana, P.J., Boeniger, M., Hayes, R., Suruda, A. and Schulte, P., Quantification of epithelial cell micronuclei by fluorescence in situ hybridization (FISH) in mortuary science students exposed to formaldehyde. *Mutat. Res.* **371**, pp. 237–48, 1996.
[33] Speit, G., Kuhner, S., Linsenmeyer, R., and Schutz, P., Does formaldehyde induce aneuploidy? *Mutagenesis* **26**, pp. 805–811, 2011.
[34] Zhang, L., Beane-Freeman, L., Nakamura, J., Hecht, S., Vandenburg, J., Smith, M., and Sonawane, B., Formaldehyde and leukemia: Epidemiology, potential mechanisms, and implications for risk assessment. *Environ Mol Mutagen.*, **51(3)**, pp. 181–91, 2009.
[35] Guyton, K.Z., Kyle, A.D., Aubrecht, J., Cogliano, V.J., Eastmond, D.A., Jackson , M., Keshava, N., Sandy, M.S., Sonawane, B., Zhang, L., Waters, M.D., and Smith, M.T. Improving prediction of chemical carcinogenicity by considering multiple mechanisms and applying toxicogenomic approaches. *Mutat Res,* **681(2–3)**, pp. 230–240, 2009.

[36] Heuser, V.D., Erdtmann, B., Kvitko, K., Rohr, P., and da Silva, J., Evaluation of genetic damage in Brazilian footwear-workers: biomarkers of exposure, effect, and susceptibility. *Toxicology*, **232(3)**, pp. 235–47, 2007.
[37] Bonassi, S., Coskun, E., Ceppi, M., Lando, C., Bolognesi, C., Burgaz, S., Holland, N., *et al.* The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN(XL)): the role of life-style, host factors, occupational exposures, health status, and assay protocol. *Mutat Res.*, **728(3)**, pp. 88–97, 2011.

ACKNOWLEDGMENTS

Começo por agradecer a todos os profissionais de Anatomia Patológica que aceitaram participar neste projeto. Obrigada também pela disponibilidade e cordialidade com que nos receberam.

Agradeço às Instituições e aos responsáveis pelos Serviços de Anatomia Patológica por terem permitido a realização do estudo.

Qualquer percurso de aprendizagem assenta numa teia de relações que envolve um vasto conjunto de pessoas e não num trabalho individual. Este espaço é dedicado a todos aqueles que contribuíram para o desenvolvimento deste estudo. A todos eles deixo aqui o meu agradecimento sincero.

In this learning journey, there were many people that helped me and gave me support, regarding my professional as well as my personal life. Thus, I would like to take this opportunity to thank:

All the individuals that agreed to participate in this study, as well as the competent authorities involved in the sensitisation campaigns.

Dr. João Paulo Teixeira, my supervisor. Thank you very much for your guidance, support, enthusiasm, advice and encouragement. It has been a pleasure to be a part of your team during these years! Thank you for believing in me frequently more than I have believed in myself! Thank you very much for everything!

Dr. Beatriz Porto, my co-adviser. Thank you for your constructive critics, for sharing your knowledge, for all the interesting discussions and great support.

To Dr. Andrew Collins, I am especially grateful for giving me the chance to work and learn with an extraordinary scientist: himself.

I also want to thank you the opportunity that you gave me to work with promising young scientists, such as Yolanda Lorenzo, Amaya Azqueta, Naouale El Yamani and Zuzana Magdolenova. To Yolanda and Marit Sandvik a very special Tusen Takk, you are extraordinary and it has been a complete pleasure to work with you.

Dr. Julia García-Léston for her availability and work even during difficult times… and also a very special GRACIAS, to the rest of the team at University of A Coruña for their precious and vast knowledge and professional support.

My gratitude to Dr. Jorge Gaspar for bringing some light into possible sources of susceptibility, and to Luís Silva Santos for his availability, work and support.

I especially want to thank the support and collaboration of Dr. Paula Guedes team (Toxicology Laboratory) and Dr. Patrícia Valentão team (Farmacognosia Laboratory) of the Faculty of Pharmacy, University of Porto, in the last phase of study.

Um agradecimento muito especial à Susana pelo incansável acompanhamento pessoal e profissional e pelo valor incalculável dos seus ensinamentos e conhecimentos. À Carla Costa e à Patrícia Coelho agradeço o acrescento tanto a nível profissional, pelo apoio laboratorial e científico decisivo para o enriquecimento do trabalho, como a nível pessoal, pela amizade sentida.

A todas as “meninas do formaldeído”, Carolina, Marta, Fátima e Sandra, muito obrigada pelo vosso trabalho e suporte nesta jornada.

A todos os elementos da família DSA-UASO agradeço a ajuda, simpatia e o excelente ambiente de companheirismo e trabalho com que me brindaram durante o período de elaboração deste trabalho. Deste fantástico grupo de pessoas preciso destacar a preciosa colaboração e profissionalismo da Paula Neves e Lívia Aguiar nas saídas de campo.

Deixo também uma nota de agradecimento aos amigos “fora do laboratório” (sabem quem são) por todo o apoio, pela compreensão nas ausências e por todos os momentos memoráveis passados, presentes e futuros.

Agradeço sobretudo à minha Mãe e Pai, e toda a família por toda a paciência e apoio durante mais esta jornada…

A todos um Muito Obrigada

Gracias

Thank You

Tusen Takk

Porto, Dezembro de 2013

Solange Costa